University of Alberta

Development of bispecific monoclonal antibodies and their applications in ultrasensitive virus ELISA; phage display technology and viral purification

By

Fei Liu

©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

in

Pharmaceutical Sciences

Faculty of Pharmacy and Pharmaceutical Sciences

Edmonton, Alberta
Spring 1999

National Library of Canada
Acquisitions and Bibliographic Services
395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque nationale du Canada
Acquisitions et services bibliographiques
395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence

Our file Notre référence

The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

L'auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

L'auteur conserve la propriété du droit d'auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

0-612-40078-6

Canada

University of Alberta

Library Release Form

Name of Author: Fei Liu

Title of Thesis: Development of bispecific monoclonal antibodies and their applications in ultrasensitive virus ELISA; phage display technology and viral purification

Degree: Master of Science

Year This Degree Granted: 1999

Permission is hereby granted to the University of Alberta Library to reproduce single copies of this thesis and to lend or sell such copies for private, scholarly or scientific research purposes only.

The author reserves all other publication and other rights in association with the copyright in the thesis, and except as hereinbefore provided, neither the thesis nor substantial portion therefore may be printed or otherwise reproduced in any material form whatever without the author' prior written permission.

3260 Ganymede Drive
Burnaby B.C. V3J 1A4
Canada

Date: Jan. 25, 1999

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled DEVELOPMENT OF BISPECIFIC MONOCLONAL ANTIBODIES AND THEIR APPLICATIONS IN ULTRASENSITIV VIRUS ELISA, PHAGE DISPLAY TECHNOLOGY AND VIRAL PURIFICATION submitted by Fei Liu in partial fulfillment of the requirements for the degree of Master of Science in Pharmaceutical Sciences.

Dr. M. R. SURESH (Supervisor)

Dr. D. E. TAYLOR

Dr. N. J. DOVICHI

Dr. D. WISHART (Chairman)

Date: Jan 25, 1999

University of Alberta

Faculty of Graduate Studies and Research

DEDICATED

to

My lovely parents, HANZHIN LIU and YUCONG HAN; my husband DI YAN and my daughter BERYL YAN with their patience and consistent support in my graduate research work.

Abstract

A monoclonal antibody (MAb) which binds to the major coat protein of filamentous phage M13 was developed by traditional hybridoma method. Subsequently a quadroma secreting anti-phage M13/anti-alkaline phosphatase (AP) bispecific monoclonal antibody (bsMAb) was developed by electro-fusion of the two parental hybridomas and selection by fluorescence activated cell sorter (FACS). In the mean time, a trioma secreting anti-M13/anti-horseradish peroxidase (HRPO) bsMAb was developed by PEG-fusion of a parental hybridoma and a spleen cell from M13 immunized mouse.

Anti-phage M13/anti-AP bsMAbs were purified by exploiting Mimetic blue A6XL affinity ligand. This unique method allows efficient bsMAb purification from parental anti-phage M13 monospecific MAbs as bsMAb-AP immune complexes under mild elution conditions minimizing damage to either antibody or enzyme activity. An ultrasensitive sandwich ELISA for detecting viruses (M13 phage as model virus) was developed using purified bsMAb-AP immune complexes coupled with an enzyme amplified ELISA. The sensitivity of the assay was increased 1000 times compared with conventional ELISA. This new ELISA format can be used to design new body fluid assays for viral load of HIV, Hepatitis and other human viruses as sensitive, rapid and inexpensive alternatives to the PCR based methods.

The anti-phage M113/anti-AP bsMAb also allowed rapid and sensitive detection of bound phage clones while panning for phages displaying peptide mimics against an antigen from a phage peptide library. Thus, this new probe could be employed in protein/peptide drug discovery by phage display technology. Furthermore, the principle of virus purification using the bsMAb as affinity ligand with a mild phosphate buffer elution was demonstrated. The results indicate that bsMAb could be used to develop affinity chromatography for purifying highly contagious and pathogenic viruses avoiding the procedures employing prolonged high speed centrifugation and multistep procedures.

ACKNOWLEDGMENTS

I would like to express my sincere and heartfelt gratitude to my supervisor Dr. M.R. SURESH, for his superb guidance and great encouragement throughout the course of this study. I will always preserve his influencing spirit of pursuing perfection and excellence.

I would like to thank my colleague F. T. KREUTZ for his valuable suggestion in techniques and my colleague D. XU for her providing of anti-alkaline phosphatase hybridoma clone and cooperative working.

Table of Contents

Chapter 1.

Introduction 1

1.1. Bispecific Antibody 1

1.1.1. History 1

1.1.2. Production of bispecific monoclonal antibody (bsMAb) 2

1.1.2.3. Chemical production 4

1.1.2.2. Hybrid hybridomas (Quadromas & triomas) 4

1.1.2.3. Genetic engineering 8

1.1.3. Purification of bsMAb 8

1.1.4. Applications of bsMAb 9

1.1.4.1. Immunohistochemistry and enzyme immunoassay 9

1.1.4.2. Immunotherapy 12

1.2. Immunoassay 13

1.2.1. Enzyme labels 13

1.2.2. Two-site (sandwich) immunometric assay 14

1.2.3. Amplified ELISA 14

1.2.3.1. Enzyme amplification ELISA 14

1.2.3.2. Tyramide signal amplification ELISA 16

1.3. Phage Display Technology 18

1.3.1. Filamentous phages 18

1.3.2. Phage display technology 19

1.3.3. Methods of screening phage displaying peptide drugs 21

1.3.3.1. Affinity purification (panning) 21

1.3.3.2. Immunological screening 22

1.3.3.2. ELISA 22

1.4. Virus Detection 25

1.4.1. Viral load 25
1.4.2. Diagnostic ELISA 25
1.5. Virus Purification 27
1.6. Aims, Objectives and Hypothesis 28

Chapter 2.
Production and Characterization of Monoclonal Antibodies against Phage M13 **30**

2.1. Introduction 30
2.2. Materials and Methods 31
2.2.1. Materials 31
2.2.2. Development of anti-phage M13 hybridomas 31
2.2.2.1. Antigen preparation 31
2.2.2.2. Immunization 33
2.2.2.3. Fusion of cells for producing anti-phage M13 hybridomas 33
2.2.2.4. Screening for anti-phage M13 hybridomas 35
2.2.2.5. Cloning and recloning of selected hybridomas 35
2.2.3. Characterization of anti-phage M13 monoclonal antibodies (MAbs) 37
2.2.3.1. Antibody isotyping 37
2.2.3.2. Western blotting for determining specificity of MAbs against phage coat proteins 37
2.2.3.3. Relative avidity analysis of anti-phage M13 hybridomas 38
2.2.3.4. Crossreactivity with fd phage 38
2.2.3.5. Crossreactivity with *E.coli* proteins 39
2.2.4. Purification of anti-phage MAbs 39
2.2.4.1. Ascites production 39
2.2.4.2. Purification of MAb 40
2.3. Results and Discussions 40

2.3.1. Development of anti-phage M13 hybridomas 40
2.3.2. Characterizations of anti-phage MAbs 45
2.3.2.1. Isotype of anti-phage M13 MAbs 45
2.3.2.2. Specificity of anti-phage M13 MAbs against phage coat proteins 45
2.3.2.3. Relative avidity analysis of anti-phage MAbs 48
2.3.2.4. Crossreactivity of anti-phage M13 MAbs with *E.coli* proteins 50
2.3.2.5. Crossreactivity of anti-phage M13 MAbs with fd phage 50
2.3.3. Purification of anti-phage M13 MAbs 51

Chapter 3.

Production and Characterization of BsMAbs **53**

3.1. Introduction 53
3.2. Materials and Methods 54
3.2.1. Materials 54
3.2.2. Hybridoma cell lines 54
3.2.3. Development of anti-phage M13/anti-AP quadromas 55
3.2.4. Screening for quadromas secreting bsMAbs 56
3.2.5. Development of anti-phage M13/anti-HRPO trioma 56
3.2.6. Screening for triomas secreting bsMAbs 56
3.2.7. Purification of anti-phage M13/anti-AP bsMAbs 57
3.2.8. Optimization of ELISA 59
3.2.9. Comparison of bsMAb, MAb and polyclonal Ab in detecting phage 60
3.3. Results and Discussions 60
3.3.1. Development of anti-phage M13/anti-AP quadromas 60
3.3.2. Development of anti-phage M13/anti-HRPO triomas 64
3.3.3. Purification of P106.5B bsMAbs 64
3.3.4. Sensitivity and background of three anti-phage antibodies in detecting phage 69

Chapter 4.

Applications of Anti-phage M13/anti-AP BsMAb in Ultrasensitive Virus ELISA, Phage Display Technology and Viral Purification **75**

4.1. Introduction 75
4.2. Materials and Methods 76
4.2.1. Materials 76
4.2.2. Antibodies and hybridomas 76
4.2.3. Development of ultrasensitive ELISA for detecting viral antigen 77
4.2.3.1. Enzyme amplified ELISA 77
4.2.3.2. Conventional ELISA 77
4.2.4. Screening phage peptide library for phage displaying biotin mimetic peptide 78
4.2.4.1. Detection of target binding phage by streptavidin capture ELISA 78
4.2.4.2. Phage standard curve by sandwich ELISA 78
4.2.4.3. Biopanning of phage peptide library 79
4.2.5. BsMAb based affinity method for purification of viruses 80
4.2.5.1. Microtiter plate method 80
4.2.5.2. Mimetic Blue absorbent column method 80
4.3. Results and Discussions 82
4.3.1. Enzyme amplified sandwich ELISA for detecting viruses 82
4.3.2. Utilities of bsMAb in improving biopanning peptide from phage peptide library 86
4.3.3. BsMAb based affinity method for purification of viruses 94
4.3.4. Conclusion

Chapter 5.

Summary, Conclusions and Future work **98**

References 102

List of Tables

Table 2.1	Immunization protocol	34
Table 2.2	Antibody response to PEG and $CsCl_2$ gradient purified M13 phages	41
Table 2.3	ELISA results of screening anti-phage M13 primary hybridoma clones	44
Table 2.4	Summary of characters of the five selected primary hybridoma clones	46
Table 3.1	ELISA results of screening anti-M13 phage/anti-AP quadromas	63
Table 3.2	ELISA results of screening anti-M13 phage/anti-HRPO triomas	65
Table 4.1	Sensitivity or lowest detection limit of enzyme amplified ELISA	84
Table 4.2	Primary data of the streptavidin capture ELISA after each amplification cycle of panning phage displaying peptide library	91
Table 4.3	(a) BsMAb affinity purification of M13 phage on microtiter plate (b) BsMAb affinity purification of M13 phage on Mimetic blue column	96

List of Figures

Figure 1.1	Schematic diagram of monospecific MAb and bsMAb	3
Figure 1.2	Schematic representation of productions of hybridma, trioma and quadroma	7
Figure 1.3	Ten possible antibody molecular isoforms produced by H and L chain combination in a hybrid hybridoma	10
Figure 1.4	(A) A two-site (sandwich) immunoassay (B) Noncompetitive immunoassay for quantification of antibodies (C) An immunoglobulin class capture assay	15
Figure 1.5	Enzyme amplified ELISA	17
Figure 1.6	Schematic representation of the phage particle.	20
Figure 1.7	Schematic diagram of engineered antibody fragments	23
Figure 1.8	Schematic representation of a typical 'biopanning' to isolate high affinity ligands from a large radom phage library	24
Figure 2.1	Schematic representation of different ELISA formats	36
Figure 2.2	SDS-PAGE and silver staining of phage M13 coat proteins	43
Figure 2.3	Western blot analysis for determining the specificity of anti-phage M13 primary hybridomas against phage coat proteins	47
Figure 2.4	Relative avidity analysis of different anti-M13 phage MAbs	49
Figure 2.5	DE-52 ion exchange chromatography purification of P93.11 MAb	52
Figure 3.1	Schematic representation of Mimetic Blue A6XL affinity purification of P106.5B bsMAb	58
Figure 3.2	(1) FACS analysis of unfused hybridomas (2) FACS analysis of fused hybridomas	62
Figure 3.3	Chromatography of the Mimetic Blue A6XL purification of P106.5B bsMAb-AP immune complexes	67
Figure 3.4	SDS-PAGE analysis of purified P106.5B-AP immune complexes	68
Figure 3.5	Comparative ELISA analysis of crude and purified bsMAb	70

Figure 3.6 Determination of optimal dilution of three types of anti-M13 phage antibodies for a streptavidin capture ELISA 71

Figure 3.7 Comparison of the three anti-phage antibodies in detecting fd phages displaying biotin mimetic peptide 73

Figure 4.1 Schematic representation of Mimetic Blue A6XL affinity purification of viruses 81

Figure 4.2. Comparison of conventional and enzyme amplification ELISA for sensitivity of detecting phage M13 83

Figure 4.3 ELISA monitoring of target binding phage in screening phage display peptide library 89

Figure 4.4 Comparison of three anti-phage Abs in detecting target binding phage in panning phage displaying peptide library 90

Figure 4.5 ELISA analysis of five selected phage clones after fourth panning and corresponding peptide sequences 93

List of Abbreviations and Symbols

A_{280}	Absorbance at 280 nm
Ab	Antibody
Ab-Ag	Antibody-antigen
ABTS	2.2'-azino-di[3-ethyl-benzthiazoline sulfonate]
AC	Alternating current
ADCC	Antibody dependent cell mediated cytotoxicity
ADH	Alcohol dehydrogenase
Ag	Antigen
AIDS	Acquired immunodeficiency syndrome
AP	Alkaline phosphatase
Aza	Azaguanine
Aza^r	Azaguanine resistant
BPTI	Bovine pancreatic trypsin inhibitor
BSA	Bovine serum albumin
BsMAb	Bispecific monoclonal antibody
CEA	Carcinoembryonic antigen
cDNA	Complementary DNA
CDR	Complementary-determining region
CMV	Cytomeglovirus
CA125	Cancer antigen 125
CTL	Cytotoxic T lymphocytes
Da	Dalton
DEAE	Diethylaminoethyl
DNA	Deoxyribonucleic acid
E.coli	Escherichia coli
ELISA	Enzyme-linked immunoassay

FACS	Fluorescence activated cell sorter
FPLC	Fast performance liquid chromatography
FITC	Fluorescein isothiocyanate
g3p	Product of gene 3
g8p	Product of gene 8
pVI	Protein 6
pIII	Protein 3
p VII	Protein 7
pVIII	Protein 8
pIX	Protein 9
H	Immunoglobulin heavy chain
HAT	Hypoxanthine, aminopterin and thymidine medium
HAT^R	HAT resistant
HAT^S	HAT sensitive
HCG	Human chorionic gonadotrophin
hGH	Human growth hormone
HGPRT	Hypoxanthine guanine phosphoribosyl transferase
$HGPRT^-$	HGPRT deficiency
HIV	Human immunodeficiency virus
HPLC	High performance liquid chromatography
HRPO	Horse radish peroxidase
HSV	Hepes simple virus
Ig	Immunoglobulin
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgG1	Immunoglobulin G1
IgG2a	Immunoglobulin G2a
IgM	Immunoglobulin M
INT	p-iodonitrotetrazolium
Kd	Kilodalton

L	Immunoglobulin light chain
LMW	Low molecular weight
MAb	Monoclonal antibody
MW	Molecular weight
NADP	Phosphorylated nicotinamide adenine dinucleotide
PAGE	Polyacrylamide gel electrophoresis
pNpp	Para-nitrophenyl phosphate
NK	Nature killer cell
°C	Degree centigrade
O.D.	Optical density
$Ouab^R$	Ouabain resistant
$Ouab^S$	Ouabain sensitiye
OPI	Oxaloacetic acid, sodium pyruvate, bovine insulin,
PBS	Phosphate-buffered saline
PBST	0.05% Tween 20 in PBS
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PSA	Prostate specific antigen
RSV	Respiratory syncytial virus
RT	Room temperature
RNA	Ribonucleic acid
scFv	Single chain Fv
SDS	Sodium dodecylsulfate
TK	Thymidine kinase
TSA	Tyramide signal amplification
TSH	Thyroid stimulate hormone
TcR	T-cell receptor
TRITC	Tetramethylrhodamine isothiocyanate
U	Units
UV	Ultraviolet

VZV	varicella-zoster virus
V/V	Volume by volume
V_H	Immunoglobulin heavy chain variable domain
V_L	Immunoglobulin light chain variable domain

Chapter 1. Introduction

1.1. Bispecific Antibody

1.1.1. History

The serum antibodies produced in response to an antigen are heterogeneous because the multiple epitopes on the antigen induce proliferation and differentiation of various B-cell clones. This antibody heterogeneity has apparent advantages in increasing immune protection *in vivo* but it limited the efficiency of an antiserum for various uses *in vitro*. Conventional heterogeneous antisera vary from animal to animal and contain undesirable nonspecific or cross-reacting antibodies. Removal of antibodies with unwanted specificity from the preparation of polyclonal antibodies can be a very time consuming task.

An alternative and simple approach is to generate pure (monospecific) clones of plasma cells *in vitro*. From these pure hybridoma clones, monoclonal antibodies (MAbs) with single antigenic specificity can be obtained. This approach was not technically feasible for many years because of the short life span of plasma cells and difficulties in maintaining them in tissue culture. In 1975, a landmark discovery was made by Kohler and Milstein (1975) in generating continuous *in vitro* cultures of fused lymphocytes secreting antibodies with predetermined specificity. By fusing a normal B cell (plasma cell) with a myeloma cell (cancerous plasma cell), a hybrid cell named as hybridoma was produced, the hybridoma not only possessed the immortal-growth properties of the myeloma cell but also secreted an antibody product of the B cells. Kohler and Milstein were awarded a Nobel prize in 1984 for this work.

Hybridoma technology opened a wide range of applications in fundamental and applied immunology. Many immunodiagnostic procedures are dependent on the highly specific interaction of the antigen intrest with an MAb carrying a measurable probe. The appropriate probe, which may be an enzyme, a fluorescent substance or a radioactive

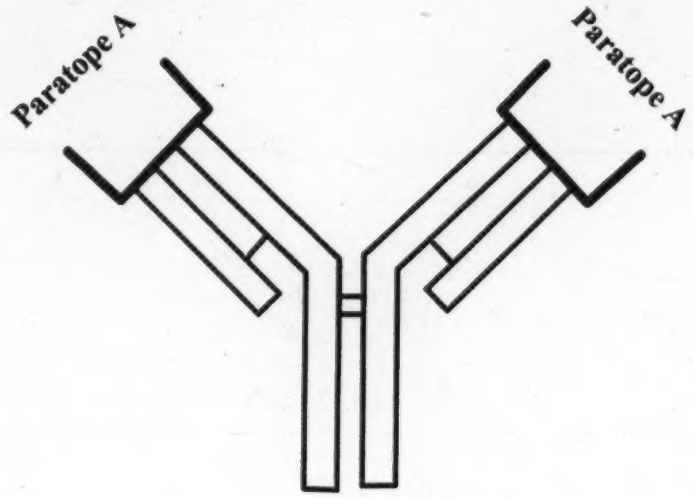
moiety, is generally linked to the monoclonal antibody by a chemical bond. MAbs have also been conjugated to chemotherapeutic agents such as toxins, drugs or isotopes for targeted immunotherapy. These targeted antibody conjugates have been used in the treatment of a number of different tumors with varying degrees of success (Marx, 1982; Cobbold and Waldmann, 1984).

Direct coupling of antibodies to diagnostic and therapeutic entities has some major disadvantages. Chemical manipulation can inactivate antibody binding sites as well as cause crucial alterations in the effector agents, thereby decreasing the efficiency of the immunoconjugates (Hurwitz et al., 1975; Ishikawa,1996). Another obstacle to the clinical use of MAbs in humans is that they are usually of mouse origin and therefore are recognized as foreign, inducing an anti-isotype response. Alternative technologies for producing MAbs have been developed since the first description of hybridoma technology. For example, bispecific monoclonal antibodies (Milstein and Cuello, 1983) and chimeric antibodies containing only mouse variable regions or CDR's (Morrison and Oi 1989; Winter and Milstein, 1991) have also been developed.

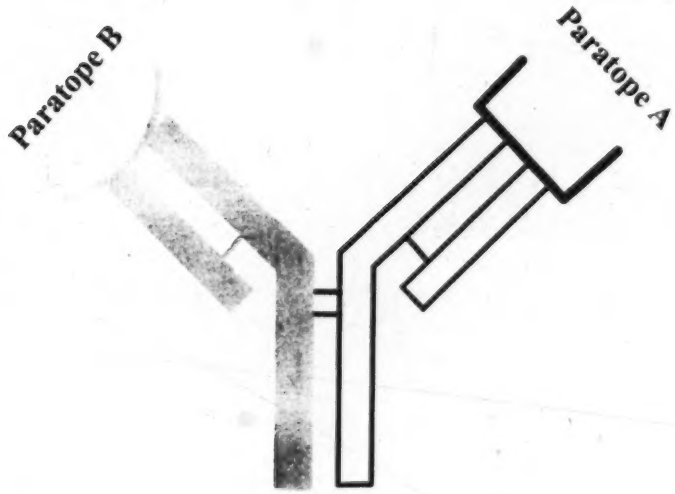
A MAb has two identical binding sites for antigen. In contrast a bispecific monoclonal antibody (bsMAb) is an antibody with two different predetermined binding specificities (Fig.1.1). This eliminate the requirement to link effector molecules chemically since one of the Fab arms is directed towards this specificity. BsMAbs were first generated nearly 40 years ago by chemical methods (Nisonoff and Rivers, 1961) but their application was limited since only polyclonal antibodies were available at that time. In the past two decades, bsMAbs as second generation of MAbs have been developed for a variety of *in vivo* and *in vitro* applications (Cao and Suresh, 1998).

1.1.2. Production of BsMAbs

BsMAbs are second generation, artificially engineered antibodies. BsMAbs can be produced mainly by three methods: (1) chemically linking two antibody molecules, (2) fusion of two different hybridomas and (3) Using recombinant DNA approuches.



Monospecific antibody



Bispecific antibody

Fig. 1.1 Schematic diagrams of monoclonal antibody and bispecific antibody.

Each of these has its advantages and disadvantages. Studies indicate that both the specificity affinities of the respective paratopes in a bsMAb molecule are similar to those of parental MAbs (Nolan and O' Kennedy, 1990).

1.1.2.1 Chemical production

Chemical coupling can be achieved through either direct coupling of whole antibody molecules (Glennie et al., 1987; Cook and Wood, 1994) or dissociation and reassociation of heterologous immunoglobulins (Nisonoff and Rivers 1961; Hammerling et al., 1968). The latter approach requires chemical manipulation to dissociate the immunoglobulins into half molecules, mixing the two antibodies and reforming the disulphide bonds linking the heavy chains. By chemical crosslinking, bsMAbs can be prepared and purified easily with high yield (Glennie et al., 1987; Cook and Wood, 1994). However, these bsMAbs have different physical and biological properties from native immunoglobulin molecules. Chemical manipulations frequently result in the formation of intrachain and mismatched disulphide bonds. The biological activity of an antibody could be decreased by altering the antigen binding sites. The 'heteroconjugates' derived by direct coupling of antibody molecules may have difficulty in penetrating the target site *in vivo* due to their size (Webb et al., 1985). BsMAbs prepared by chemical linking have generally been used for *in vitro* applications and are less suitable for animal or human studies (Webb et al., 1985).

1.1.2.2. Hybrid hybridomas (Quadromas & triomas)

Hybrid hybridomas are an extension of the hybridoma technique developed by Milstein and Kohler in 1975 (1975). This technique was based on the fact that a normal B cell producing antibody molecules of a single specificity was not able to grow in tissue culture while on the other hand, many tumor cells were capable of growth indefinitely in tissue culture. So fusion of an antibody-producing cell with a myeloma cell (tumor cell) would result in a hybrid cell (hybridoma) with both properties of secreting antibodies and

mmortal growth. With the use of hybridomas, it was possible to produce large quantities of a specific antibody.

When Sendai virus or polyethylene glycol are used as fusion agents, only a small percentage of the cells actually fuse. Some of these fused cells are so-called homofusions A-A (two myelomas) or B-B (two B cells) rather than the desired A-B hybrid. In order to isolate the desired they must be separated from fused or unfused myeloma cells. Without separation, the myeloma cells would overgrow the hybridomas.

HAT selection is the most widely used method for selecting fused cells. HAT selection depends on the fact that mammalian cells can synthesize nucleotides by two different pathways, i.e., the *de novo* and the salvage pathways. In the *de novo* pathway, cells can use a methyl or a formyl group transferred from an activated form of tetrahydrofolate to synthesize nucleotides. When the *de novo* pathway is blocked by *aminopterin* (a folic acid analog), cells will use the salvage pathway, which by passes the *aminopterin* block by converting purines and pyridines directly into DNA and RNA. The enzymes involved in the salvage pathway include hypoxanthine-guanine phosphoribosyl transferase (HGPRT) and thymidine kinase (TK). A mutation in either of these two enzymes can block the salvage pathway. HAT medium contained *aminopterin* to block the *de novo* pathway, as well as hypoxanthine and thymidine to allow growth of cells via the salvage pathway.

SP2/0 is a mutant myeloma cell line lacking HGPRT and is commonly used for the generation of hybridomas. The SP2/0 cells cannot incorporate hypoxanthine and hence would die in HAT medium. On the other hand, the hybrid cells have the wild type HGPRT enzyme from the parental B cell, and therefore survive in HAT medium. Further the unfused or fused spleen cells do not need to be weeded out because they are terminal cells of a differentiation series and are only capable of limited growth *in vitro*.

If two hybridomas are fused, the hybrid cell (hybrid hybridoma or quadroma) would produce a combination of immunoglobulins, including bsMAbs (Milstein and Cuello, 1983). Once a hybrid hybridomas is established, it can serve as an immortal cell to produce endless amount of bsMAbs, in the same way as hybridomas can produce edless amounts of MAbs. An alternative approach can be used to produce bsAMbs in

which a hybridoma with specificity for one antigen can be fused with a spleen cell from an animal immunized with the second antigen. The resulting somatic hybrid is referred to as a trioma, since it was derived from three parental cells (Webb et al., 1985; Suresh et al., 1986b). Both methods of deriving quadromas and triomas are based on the conventional hybridoma technology (Fig.1.2).

The hybridomas used for both quadroma and trioma fusions are commonly back-selected for HAT sensitivity prior to further fusion since these hybridomas were originally resistant to HAT media. This procedure can be carried out by growing the cells in a complete growth medium with increasing concentrations of 8-azaguanine (Kontsekova et al.,1991) thus inducing a HGPRT deficiency. For quadroma fusions, HAT sensitive hybridomas can be further modified for resistance to biological inhibitors or cytotoxic drugs. Ouabain is one of the most popular cytotoxic drugs for selecting resistant cells (Baker et al., 1974; Suresh et al., 1986b; Chervonsky et al., 1988). Uses of emetine, actinomycin D and neomycin have also been reported (Suresh et al., 1986b Chervonsky et al., 1988; Wim et al., 1989). Thus, quadromas with both HAT and ouabain resistance would be produced when a HAT-sensitive-ouabain-resistant hybridoma is fused with a HAT-resistant-ouabain-sensitive hybridoma (wild-type).

Although there have been quite a few reports (Tada et al., 1989; Kontsekova et al., 1991) generating triomas, many researchers are concentrating on quadroma production, the fusion of two hybridoma cell lines. It is potentially easier to work with a population of cells producing antibodies of known antigenic specificity and affinity than to work with a heterogeneous population of spleen cells producing uncharacterized antibodies.

Selection of drug sensitive or drug resistant hybridoma parental cell lines is a labor-intensive procedure. A new method (Karawajew et al., 1987) was developed to eliminate the laborious and time consuming steps of selecting drug sensitive/resistant hybridoma clones. The two parental hybridoma cells used for fusion are labeled with either fluorescein isothiocyanate (FITC) or tetramethylrhodamine isothiocyanate (TRITC). Differentially labeled hybridomas are then fused and cells containing both FITC and TRITC can be selected by a fluorescent activated cell sorter (FACS) without relying on drug selection.

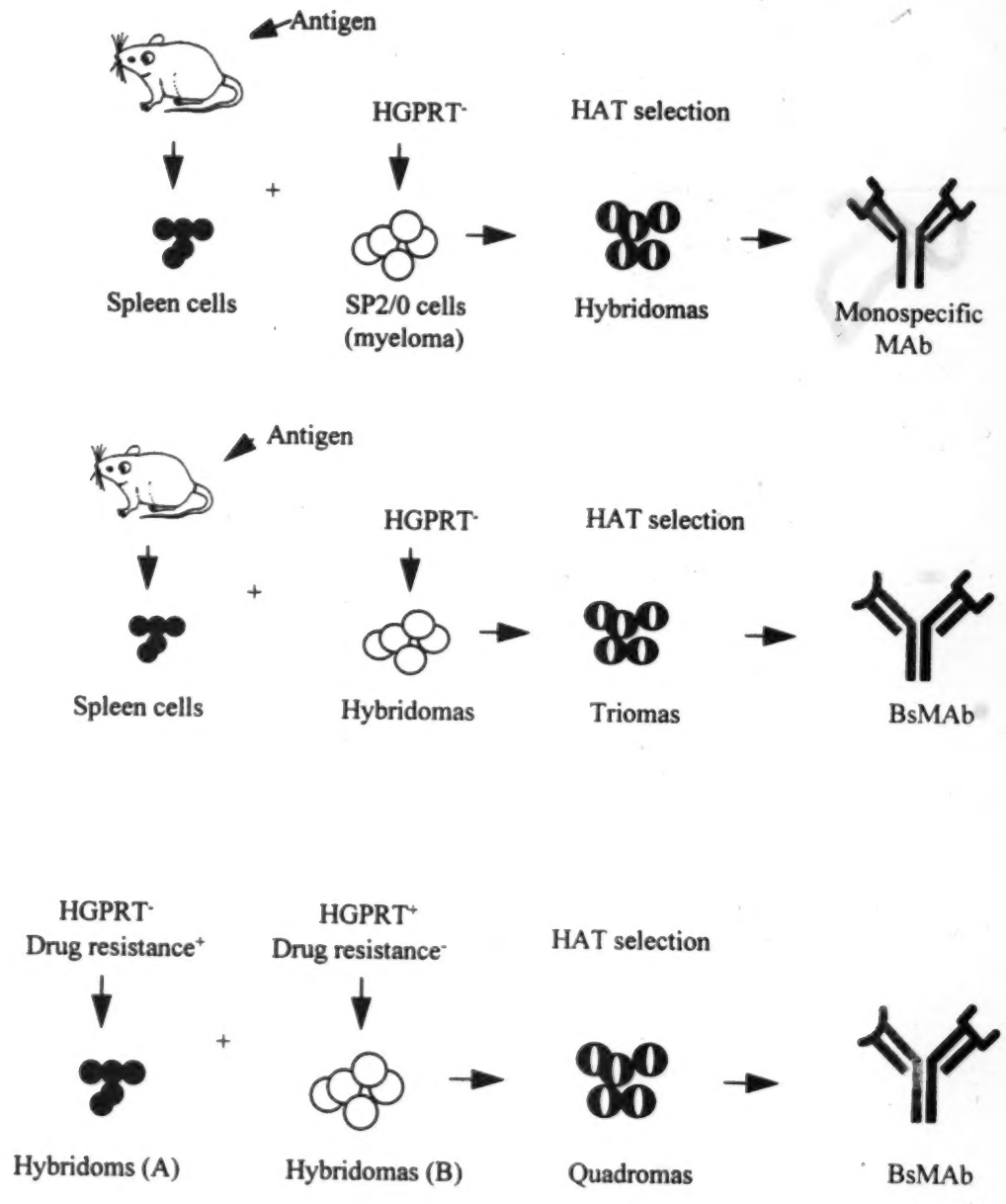


Fig. 1.2 Productions of hybridomas, triomas and quadromas

1.1.2.3. Genetic engineering

Two major disadvantages can limit the applications of the bsMAbs produced by hybrid hybridomas. Firstly, hybrid hybridomas generate a heterogeneous mixture of antibodies. The purification of the bsMAb from the other homo and hetero immunoglobulin species is labor intensive. Secondly, the current clinical use of bsMAbs are based mostly on murine antibodies, which can elicit human anti-mouse antibody responses eventually resulting in the formation of immune complexes and rapid clearance of bsMAbs from circulation. The limitations of generating bsMAbs through these traditional methods may be potentially overcomed by genetic engineering techniques (Staerz and Bevan, 1989).

The advances in the molecular cloning of MAbs have provided important techniques that may be adapted to the production of bsMAb. One of the first descriptions of genetically engineered bsMAb constructs was given by Songsivilai et al., (1989). Two different chimeric sequences were co-transfected into a murine cell line, producing humanized whole molecule bsMAbs. BsMAb molecules have also been made as a single covalent structure by combining two single chain Fv (scFv) fragments using a polypeptide linker (Winter and Milstein, 1991; Holliger et al., 1993; Kranz et al., 1995; Thirion et al., 1996). Recently, tetravalent bsMAbs with the human IgG Fc region were generated by fusion of the DNA encoding a single chain antibody after the C terminus or after the hinge with an antibody of different specificity (Coloma and Morrison, 1996). These approaches reduce human anti-mouse antibody response and yield a homogenous product with increased avidity and specificity.

1.1.3. Purification of BsMAb

Hybrid hybridomas, with the antibody gene from both hybridomas secretes not only bsMAbs but also antibodies with monospecific binding capabilities of both fusion parents (Milstein and Cuello, 1983). Up to 10 different antibody molecular species (Fig.1.3) can be produced, theoretically, in a hybrid hybridoma by a total radom

association of two heavy, H_1H_2 and two light, L_1L_2 chains (Suresh et al., 1986a). Monospecific MAb with mono-or bi-valency can compete against the bsMAb for the antigen binding sites. Further, non-homologous associations (H_1L_2 or H_2L_1), in one or both binding sites. can potentially interfere with the specificity of the antibody by creating a completely new paratope. Hence, purification of bsMAb is highly desirable for their applications in immunoassays and immunotherapy.

Ion exchange chromatography (DEAE) has been traditionally used to purify the bsMAbs of different subclasses produced by a hybrid hybridoma (Suresh et al., 1986 a & b; Warnaar et al., 1994). Other ion-exchange methods used for purifying bsMAbs include HPLC matrix, Abx (Allard et al., 1992) and FPLC matrix, Mono Q (Auriol et al., 1994). Unfortunately, ion exchange chromatography lacks complete specificity and can't completely remove all MAbs and undesired antibody species.

Dual affinity chromatography (Kuppen et al., 1993; Cao and Suresh, 1998) can produce the most pure form of bsMAb. This method is based on sequential antibody interaction with two different antigens immobilized on two separate columns. The bsMAb sample purified through both the antigen affinity columns contains only hybrid molecules presenting intact paratopes against both antigens. Since a low pH (2.8-4) buffer is normally required to elute the bound antibody on the column, bsMAbs are often easily denatured. New methods to purify bsMAbs are needed to improve the purification of bsMAb in terms of quality, yield and simplicity.

1.1.4. Applications of Bispecific Monoclonal Antibody

1.1.4.1. Immunohistochemistry and enzyme immunoassay

BsMAbs were designed to specifically cross-link the antigen with a signal-generating enzyme, eliminating the need for chemical conjugation of the enzyme to the antibody. Chemical conjugation has many disadvantages including (1) Chemical cross-linking is random, generally resulting in batch to batch variations in reagent quality, leading to variable backgrounds. (2) The size of conjugates or complexes obtained is

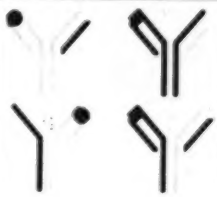
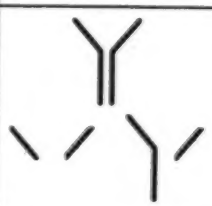
Heavy (H) and Light (L) chain recombination	Bispecific	Monospecific	Presumed inactive
Two homologous H-L associations & heterologous H_1-H_2 combination			
Parental H-L associations		bivalent	
One non-homologous H-L associations		 monovalent	
Two non-homologous H-L associations			

Fig. 1.3 Ten possible antibody molecular isoforms produced by random H and L chain conbination in hybrid hybridomas (Modified from Suresh et al., 1986a).

not uniform and this varability influence their penetration in immunohistochemical applications. (3) Active site or binding site inactivation of the markers and/or the immunoglobulin may occur. These drawbacks associated with covalent cross-linking of antibodies to enzymes or radioactive markers often influences the linearity and reproducibility of an assay method (Suresh, 1991). It has been suggested that these problems associated with chemical conjugates may be overcome by the use of bsMAbs.

In the earlier studies of Milstein and Cuello (1983), these two researcher developed a bsMAb (anti-somatostain x anti-peroxidase) and utilized it in an immunohistochemistry assay. Suresh and co-workers (1986 a & b) established a hybrid-hybridoma secreting anti-peroxidase x anti-substance P bsAMb, which was used in immunocytochemistry and exhibited major improvements in sensitivity, signal-to-noise ratio, simplification of staining procedures and an unusually uniform staining pattern across entire tissue section with ultrastructural preservation of subcellular element.

More recently, many investigators have developed bsMAbs directed against an enzyme (e.g. horse radish peroxidase, alkaline phosphatase or ß-galactosidase) and a second antigen (e.g. tumor specific antigen, peptide or hormone) for use in enzyme immunoassay or immunohistochemistry. A high sensitivity bsMAb immunoassay for HCG has been described, using a bsMAb binding HCG and urease to detect HCG levels of only 25 mIU/mL (Takahashi and Fuller, 1988). A homogeneous enzyme immunoassay has also been developed using a bsMAb (Gorog et al., 1989). This BsMAb was produced by reacting with both CEA (carcinoembryonic antigen) and ß-galactosidase. Antibody-mediated protection against thermal denaturation allows the free enzyme to be destroyed by heating at 62^0C for 1 hr. Substrate can be added directly to the solution to quantify the remaining enzyme. Under appropriate conditions, heat-resistant enzyme activity was shown to be proportional to concentration of CEA in the range up to 75 ng/mL, the range likely to be of clinical significance. A bsMAb anti-TSH (thyroid stimulating hormone) x anti-AP has demonstrated greatly reduced non-specific binding and an improved detection limit in the TSH assay compared with covalently linked AP-MAb conjugate (Ishikawa, 1996). More recently, an anti-CA125 (cancer antigen 125) x anti-peroxidase bsMAb (Kreutz and Suresh,1995) and an anti-PSA (prostate-specific antigen) x anti-

peroxidase bsMAb (Kreutz and Suresh 1997) demonstrated high specificity, excellent detection limits, and fast kinetics in developing single-step assay for detection of these tumor specific antigens.

A recent study has shown that bsMAbs could be used in biosensors (Reiken et al., 1996). The nicotinic acetylcholine receptors reconstituted in bilayer lipid membranes were inactivated when two bsMAbs, attached to the same receptor, bind to a single antigen molecule. Experiments with patch clamp recording equipment revealed that antigen levels of 10^{-8} M completely and irreversibly inactivated small numbers of nicotinic acetylcholine receptors. This approach may lead to the construction of biosensors capable of detecting individual antibody-antigen (Ab-Ag) binding events.

1.1.4.2. Immunotherapy

Radioimmunotherapy with MAb conjugates is severely limited by radiation toxicity to normal organs, especially blood, liver, kidney and bone marrow. This is largely due to the high radiation dose from prolonged circulating radiolabeled antibody to those organs, from normal tissue accumulation of the antibody, or from free radionuclide deposited in the organs. This problem could be overcome by a two-step targeting technique with a BsMAb. In particular, a bsMAb could be constructed to recognize a tumor antigen and a radioisotope (^{99m}Tc, ^{90}Y, ^{67}Ga, ^{111}In). The non-radiolabeled antibody with slow uptake kinetics is initially localized to tumor, and excess antibody is given time to clear from the circulation. Then the small radiolabeled hapten with fast kinetics is administered that binds to the bsMAb already taken up by the tumor target (Chetanneau et al., 1994; Chatal et al., 1995 and Schuhmacher et al., 1995).

BsMAbs also are very useful in delivery of cytotoxic compounds, drugs and toxins not only because they have the advantages in two step delivering but also neither the antibody nor the cytotoxic molecule is inactivated as a result of conjugation. In addition, no covalent bonds would have to be broken to achieve full cytotoxic potency once the complex has reached its target. BsMAbs have been made to specifically deliver methotrexate (Affleck and Embleton, 1992), saporin (Bonardi et al., 1992; French et al.,

1995), Doxorubicin (Reddy and Ford, 1993) and vinca alkaloids (Corvalan et al., 1987) to the appropriate targets.

The Fc end of an IgG antibody binds to FcγR on effector cell or molecule with far less discrimination and potentially links target cells to FcγR on platelets, B cells or other non-cytotoxic cells. Furthermore, a MAb with appropriate target specificity may not mediate cytotoxicity if it is of an isotype which does not activate human complement and/or bind well to FcγR on human cells. BsMAbs have been developed to bind both to a target cell (pathogen or tumor) and to a toxin, enzyme or cytotoxic trigger molecule on a T cell such as the T-cell receptor (TcR) or FcγR. BsMAbs can thus redirect T-cell function. Many studies have been done on T-cell targeted cytotoxicity (Pere et al., 1985; Demanet et al., 1991; Renner et al., 1994). BsMAbs have also been actively investigated for targeting natural killer (NK) cells for the treatment of cancer and infectious disease (Uggla et al., 1989; Ferrini et al., 1991; Valone et al., 1995).

BsMAbs represent powerful new therapeutic tools with promising prospects for clinical uses. Advanced molecular biology techniques could be used to engineer bsMAbs for such requirements by refining the molecule to reduce size and immunogenicity.

1.2. Immunoassays

1.2.1. Enzyme Labels

The detecting antibodies used in immunoassays can be labeled with an enzyme, biotin, radioisotope or fluorescent probe. Enzymes are the most versatile and popular class of labeling substances used for immunoassays. Many are measurable at very low concentrations by utilizing their catalytic properties to generate colored, fluorescent or luminescent compounds from an appropriate substrate. A single molecule of enzyme may cause the conversion of many molecules of substrate, increasing the strength of the signal, and hence the sensitivity. In 1990, the most common enzyme label in immunoassays was horseradish peroxidase (HRPO), with alkaline phosphatase (AP) in second place (Gosling, 1990). A perusal of current issues of relevant journals confirms that this is still

true and no other enzyme seems likely to challenge their positions in the near future. HRPO reacts with hydrogen peroxide which becomes oxidized. It then oxidizes another substrate, forming a colored, fluorescent or luminescent derivative, depending on the substrate used. AP catalyzes the hydrolysis of phosphate ester of primary alcohols, phenols and amines generating detectable end-points (Wild, 1994). The substrate reaction times are generally between 10 and 30 min (Porstmann and Kiessig 1992). Horseradish peroxidase as a marker enzyme, with both chromogenic and fluorogenic products, produces a higher sensitivity in two-site ELISAs then alkaline phosphatase under identical concentrations of immune reaction (Porstmann, et al., 1985).

1.2.2. Two-site (sandwich) Immunometric Assay

There are several configurations for noncompetitive immunoassays (Fig.1.4). The two-site immunometric assays (Christopoulos and Diamandis, 1996) that will be extensively used in this thesis is described here. This assay is applied to the determination of macromolecular antigens, where simultaneous binding of two antibodies to the antigen (Fig.1.4A) is allowed without steric hindrance. In this assay, the sample is added to a solid phase, which is coated with an excess of purified anti-analyte antibody (capture antibody) and blocked. During the subsequent incubation, the capture antibody binds the analyte specifically. All other sample components are washed out and the bound analyte is then quantitied in a second step by adding an excess of labeled anti-analyte antibody (detection antibody). After incubation the unbound antibody is washed out and the signal from the solid-phase-bound detection antibody is directly related to the analyte concentration in the sample.

1.2.3. Amplified ELISA

1.2.3.1. Enzyme amplification ELISA

In a conventional enzyme-immunoassay, the enzyme label is responsible for the conversion of a substrate into a product that can be readily detected. At very low enzyme

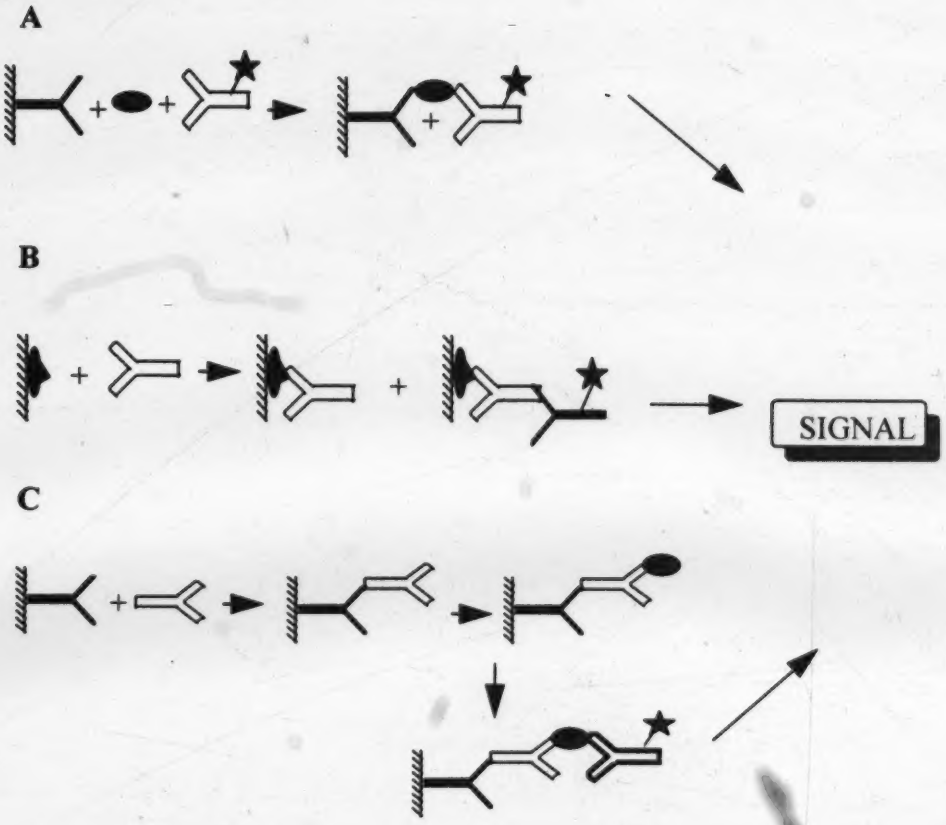


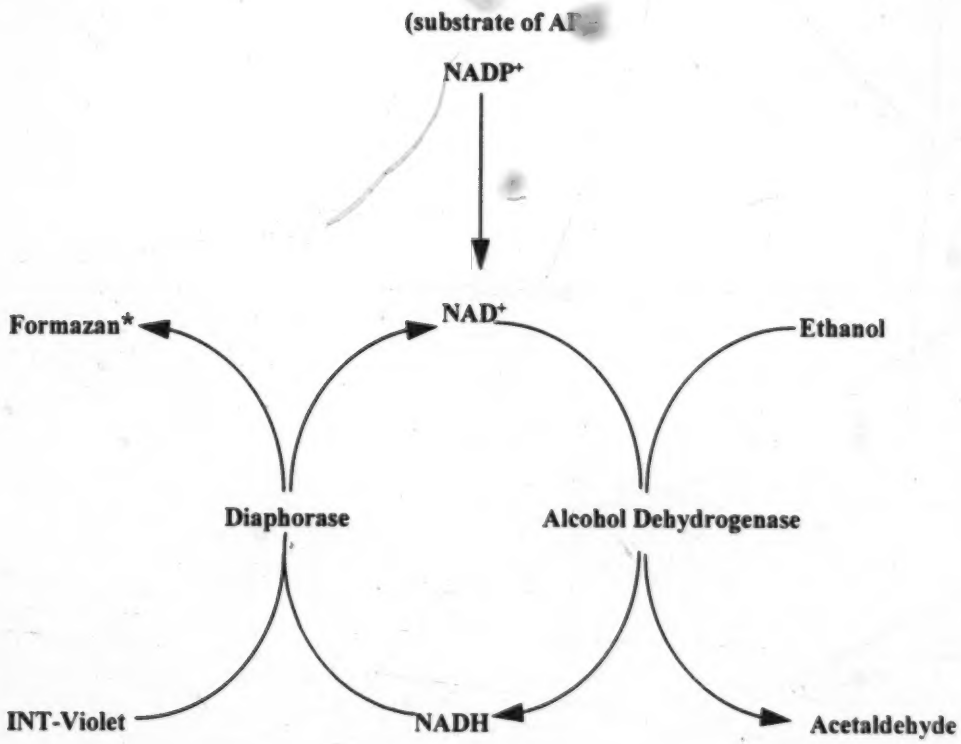
Fig. 1.4 (A) A two-site (sandwich) immunoassay. The analyte is captured by the immobilized antibody and is then detected by using a labeled antibody. (B) Noncompetive immunoassay for quantification of antibodies. The sample is incubated with an antigen-coated solid phase. The bound antibodies are then quantified by using a labeled anti-immunoglobulin. (C) An immunoglobulin class capture assay. All the immunoglobulins of the class of interest are first captured on a solid phase which is coated with anti-class antibodies. Then the antigen is added and binds only to specific antibodies of the class. The bound antigen is quantified by using a labeled antibody (refer to Christopoulos and Diamandis, 1996).

concentrations, hardly any product is generated, resulting in a weak signal that is difficult to measure because of background noise. It is possible to increase the amount of detectable product generated by using several enzymes and substrates in a cyclical fashion. The first stage of the signal generation process is conventional: the enzyme label catalyzes the conversion of the first primary substrate into product. This product is not the detectable end point, but instead, it participates along with two recycling enzymes to convert the primary product to enter a cyclical reaction to generate several hundred secondary products which may be colored, fluorescent or luminescent.

An enzyme amplification ELISA has been described (Self, 1985) using alkaline phosphatase as the label, and NADP as the primary substrate. The substrate is converted to NAD in the initial reaction which then enters a redox cycle. Diaphorase and alcohol dehydrogenase are recycling enzymes of this redox cycle and formazan is produced as the colored product of this regenerative reaction (Fig.1.5). In recent years, such detection systems have been applied for developing highly sensitive ELISAs for thyrotropin (Clark and Price, 1986) and for human proinsulin (Dhahir et al., 1992).

.2.3.2. Tyramide signal amplification (TSA)

Tyramide signal amplification (TSA) has also been used for developing sensitive immunoassays. This technique uses horseradish peroxidase to catalyze the deposition of biotin- or fluoresent-labed tyramide into tissue sections, or cell preparations previously blocked with proteins. The reaction is quick (less than 10 min) and results in the deposition of numerous biotin or fluoresent labels. These labels can then be detected by standard techniques. The TSA could enhance detection limits up to 1000-fold without loss of resolution. TSA method has been used for detecting cell surface marks such as CD8 and CD25 on tonsil tissue (Chao et al., 1996) and HIV p24 antigen in brain tissue (Strappe at al., 1997) by immunohistochemistry. The sensitivity of antigen detection is significantly increased compared to the conventional techniques. Recently the TSA method has also been applied for developing sensitive ELISA for determining viral load of HIV patients (Boni et al., 1997).



*Colored end product

Fig. 1.5 Enzyme amplified ELISA (refer to Bates, 1987).

1.3. Phage Display Technology

1.3.1. Filamentous Phages

Fd, f1, and M13 are a group of related viruses which are only able to infect bacterial strains that express sex pili encoded by an F factor (Marvin et al., 1974). Their particles are similar in size and shape. Their genomes are organized in a similar fashion, and except for a small number of base changes, their DNA sequences are identical. Fd (f1 and M13) is about 900 nm long and 6 to 10 nm thick, with a molecular weight of 12 x 10^6 Da (Berkowitz and Day, 1976). The fd (f1/M13) coat consists of 2700 to 3000 copies of a major protein, the gene 8 product and four minor components including the products of genes 3, 6, 7, and 9 numbering about five copies each (Newman et al., 1977). The minor coat proteins are located exclusively at the ends of the particle (Fig.1.6). Exploiting standard electrophoretic techniques, g3p appeared to be the only additional protein of the phage coat besides the major coat protein (Henry and Pratt, 1969). The g8p is 50 amino acids long, with a molecular weight of 5,200 Da. The g8p is capable adopting two distinct conformations in response to its respective environment: an almost 100% α-helical secondary structure when protein is integrated in the phage coat and the 50% α-helix state present in membranes, phospholipid vescles, deoxycholate micelles, or certain detergents (Boeke, 1981; Nozaki, et al 1978). In 10 mM deoxycholate, the protein is present as a stable dimer (Nozaki, et al 1978). Solid-state nuclear magnetic resonance of the g8p protein solubilized in SDS micelles proved the 50% α-helical conformation is stable in SDS (Opella, et al., 1980). The molecular weight of the g3p has been calculated from the nucleotide sequence to be approximately 43,000 Da. In polyacrylamide gel, considerable variations in MW have been observed. This is probably the consequence of several glycine/serine clusters in its sequence which may reduce the amount of SDS associated with the protein, thus decreasing its electrophoretic mobility. The apparent MW values in the polyacylamide gel range from 56 to 70 Kd (Goldmith and Konigsberg, 1977).

1.3.2. Phage Display Technology

Phage display technology is an efficient method and prolific means for producing large numbers (hundreds of millions) of diverse peptides and proteins, and selecting these molecules that perform useful functions. This is often referred to as "biopanning".

Since replication of DNA or assembly of the phage is not constrained by the size of DNA, filamentous phage is an excellent cloning vector. The insertion of foreign DNA into a non-essential region merely results in a longer phage particle. Thus, large quantities of single-stranded DNA containing foreign DNA inserts ranging from a few to thousands of nucleotides can be easily obtained (Webster, 1996).

George Smith first showed in 1985 that the linkage between phenotype and genotype could be established in filamentous bacteriophage and gave birth to the new technology of phage display. That foreign DNA fragments can be inserted into g3p of filamentous phage to create infective "fusion phage" that display foreign peptides on their surface. The peptide inserts are accessible to antibodies, allowing purification of the phage and identification of the peptide sequences they carry (Smith, 1985; Parmley and Smith, 1988). Subsequently, the screening of a small, model epitope library was reported (Scott and Smith, 1990).

The power of using phage particles for display of diverse peptide libraries is that the system permits rapid library construction, affinity selection, amplification and analysis of results. For example, 10^7-10^9 different variants can be constructed simultaneously and then winnowed down to the one or few with binding and specificity properties of the greatest interest. All of these features provided the molecular biologist with ready and general tools for ligand discovery. Ligands identified in this manner interact with the natural binding site on the target molecule (such as enzyme, receptor or antibody) and often resemble the target's natural ligand (Sparks et al., 1996). This allows one to study ligand-target molecule interactions that may enable the development of new drugs based upon these peptideleads.

In recent years, screening phage peptide library has resulted in the identification of peptides that bind to concanavalin A (Scott, 1992); the critical residues in calmodulin-binding sequences (Dedman et al., 1993); a peptide antagonist to the thrombin receptor (Doorbar and Winter, 1994) and the distinct ligand preferences of SH3 domains (Sparks et al., 1996a).

In addition to its use as a vector for accessible expression of peptide libraries, M13 has been used to display mutant libraries of entire proteins. Antibodies were the first functional proteins to be displayed on the surface of phage (Macafferty et al., 1990). All

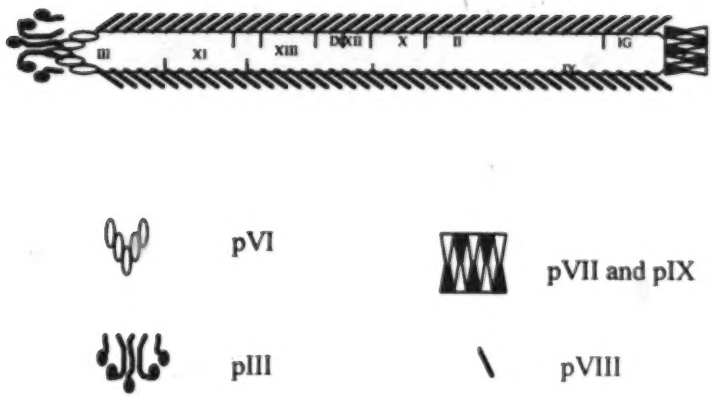


Fig. 1.6 Schematic representation of the phage particle showing the location of the capsid proteins (adapted from Webster, 1996)

three forms (Fv, scFv and Fab) of antibody variable domain fragments (Fig.1.7) have been expressed on the surface of phage (Macafferty et al., 1994).

This phage display system combined with PCR cloning of variable region genes has generated libraries of variable domain sequences that have been used to isolate novel human Abs from immunized or nonimmunized B cells (Barbas et al., 1992; Marks et al., 1991).

Other proteins to be expressed on the surface of phage include human growth hormone(hGH) (Bass et al., 1990), Bovine pancreatic trypsin inhibitor (BPTI) (Markland et al., 1991) and alkaline phosphatase (McCafferty et al., 1991). A series of studies were performed on hGH-constructed libraries of hGH with key positions randomized. Selection allowed the in vitro evolution of hGH to create a molecule with picomolar affinity for its receptor (Lowman and Well, 1993). In the case of BPTI, randomization and selection from phage display libraries was used to generate an inhibitor with a new specificity for human neutrophil elastase (Roberts et al., 1992). The ability to improve affinity and alter specificity of protein molecules with little or no knowledge of molecular structure represents an extremely powerful application of phage display technology.

1.3.3. Methods for Screening Phage Displaying Peptide Drugs

1.3.3.1. Affinity purification (panning)

Affinity purification of phage was firstly described by Parmley and Smith (1988) and has become the most common method for selecting phage displaying the desired peptide affinity to a target molecule. Affinity purification involves the following steps (Fig.1.8): (1) incubating phage with biotinylated Ab (or other targets); (2) diluting the phage target mixture and placing it on a streptavidin-coated peri dish, therefore specifically attaching Ab-reactive phage to the plastic surface through the Ab-biotin-streptavidin bridge; (3) washing away nonbinding phage remaining in solution; (4) eluting bound phage in acid; (5) propagating the eluted phage by infecting *E.coli*; and (6)iterating this process until the binding phage are purified to homogeneity. One round

of affinity purification with a target immobilized on ELISA plates typically results in an ~10^3-fold enrichment of binding over nonbinding phage. However, the rate of enrichment will vary from target to target (Devlin et al.,1990).

1.3.3.2. Immunological screening

The immunological colony screening technique was originally described by Sambrook et al. (1989) for screening cDNA libraries constructed in plasmids and the bacterophage λ expression vector. This method was adapted for screening phage displaying peptide libraries by several others (MaCafferty et al., 1990; Christian, et al., 1992).

In this method, clones from each round of affinity selection are plated on LB medium with a proper antibiotic plate, at a density wherein colonies are well separated. The colonies are then transferred to nitrocellulose membrane filters. The filter is washed and blocked. The antibody that is used for screening is then incubated with the filter. After washing, a secondary antibody is incubated with filter for detecting positive colonies.

1.3.3.2. ELISA

ELISAs that detect the interaction of antibody and peptide on phage may take two different forms (Valadon and Scharff, 1996): (1)a direct ELISA in which the phage is first immobilized to the plate by adsorption or capture with an anti-phage antibody and the antibody used for screening is then added; or (2) a reverse ELISA in which the antibody used for screening is first immobilized on the plate and then phage is added followed by the anti-phage antibody.

Phage ELISAs have been employed in many phage screening experiments to charaterize the binding of individual phage isolates with the target used for screening phage displaying a peptide library. In earlier studies on screening phage displaying peptide libraries, polyclonal antisera was used for the phage ELISA (McCafferty et al.,

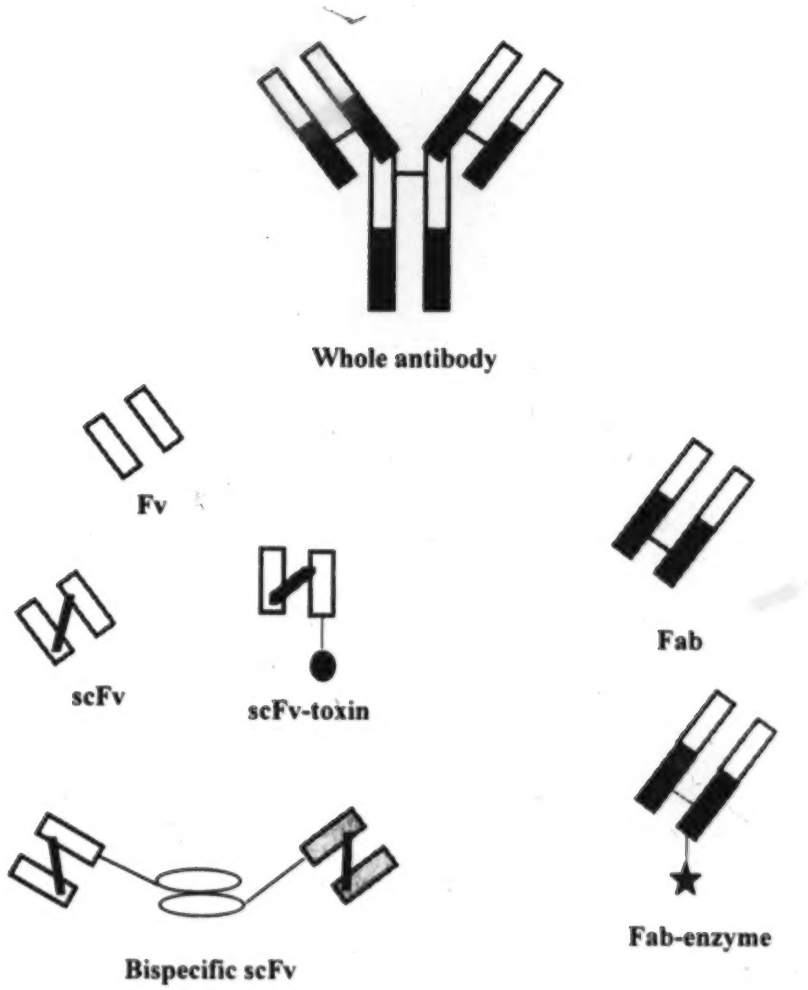


Fig. 1.7 Schematic representation of engineered antibody fragments

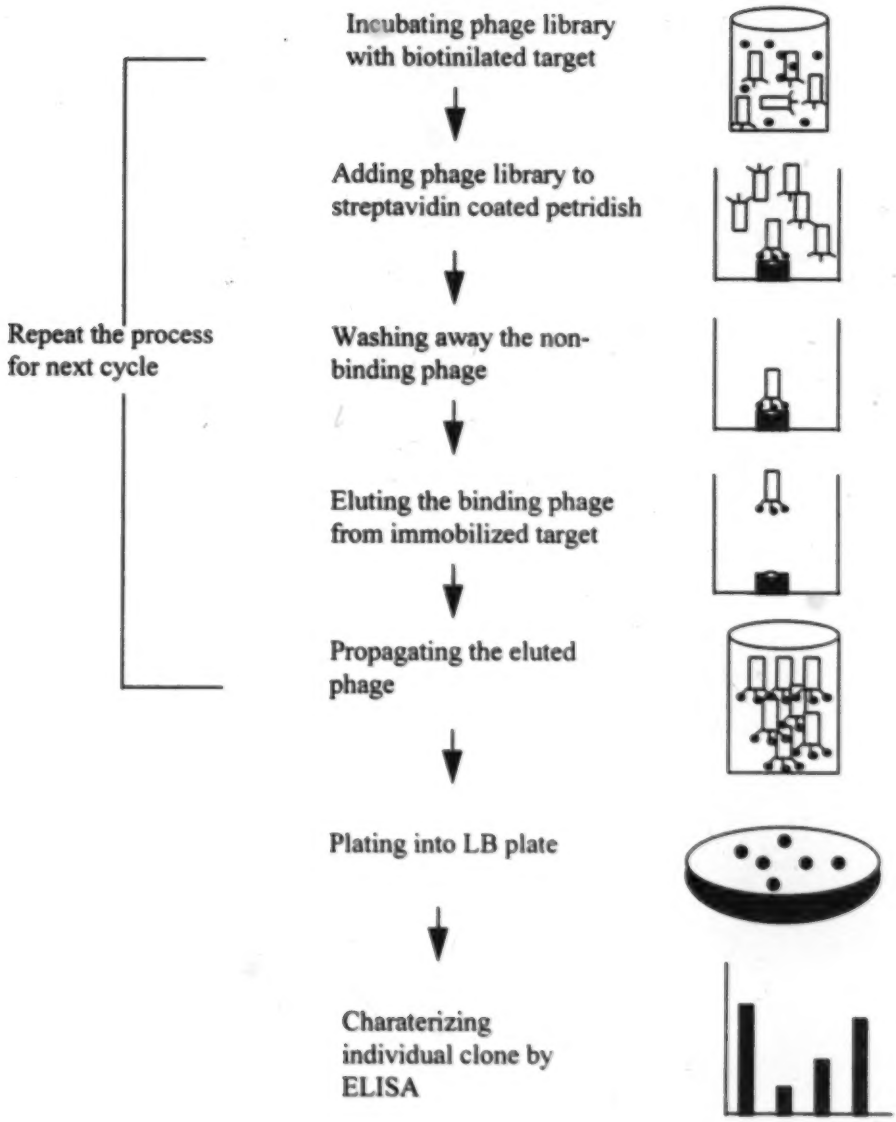

Incubating phage library with biotinilated target
Adding phage library to streptavidin coated petridish
Washing away the non-binding phage
Eluting the binding phage from immobilized target
Propagating the eluted phage
Plating into LB plate
Charaterizing individual clone by ELISA
Repeat the process for next cycle

Fig. 1.8 A typical 'biopanning' to isolate high affinity ligands from a large radom phage library

1991). Recently, an anti-phage M13 monospecific MAb has been developed (Dente et al., 1994; and Bhardwaj et al., 1995) for the same purpose.

1.4. Virus Detection

1.4.1. Viral Load

Virus load is the amount of virus in the blood (Ho 1996). Measuring virus load is useful in determining the prognosis of the infected individual and in monitoring the effectiveness of therapies. The quantification of hepatitis C virus in plasma has become a valuable diagnostic tool for the evaluation of patients undergoing treatment with interferon and in the subsequent monitoring of their response (Davis,1994). Some studies have described a decreased likelihood of achieving a long-term response to interferon treatment in individuals with a high plasma pretreatment virus load (Mita et al., 1994; Arase et al., 1995). In recent studies on AIDS (Mellors et al., 1996 and 1997), plasma viral load was found to be a better indicator of prognosis than the CD^+ lymphocyte count. Plasma viral load strongly predicts the rate of decrease in $CD4^+$ lymphocyte count and progression to AIDS and eventually death.

Measuring the virus load in infected individuals requires highly sensitive methods. In the case of HIV, virus levels can be lower than 500 copies/mL (Mollors et al., 1997). Current viral load assays depend on measurement of HIV RNA (Barbara and Klaus, 1996). The sensitivity of ELISA for virus antigen assay such as p24 antigen (Piatak et al, 1993), a marker of HIV expression, at the protein level is too low to be used for this purpose although it is simpler and more economic compared with an RNA based assay.

1.4.2. Diagnostic ELISAs

Since the introduction of the enzyme-linked immunosorbent assay (ELISA), diagnostic virology has advanced greatly (Polin et al., 1984). In the past, direct

demonstration of the organism was only possible using slow and expensive methods (tissue culture and animal inoculation). Standard culture techniques require from 48 hr up to several weeks for the isolation and positive identification of the offending virus (Polin et al., 1984). In the early stage of an infection or when healing has begun, the amount of viable virus is probably diminished and positive culture results are less likely. In addition, some viruses such as HSV, rotavirus, and RSV, cause infections that yield high levels of virus in clinical specimens but either do not replicate well in the usual cell cultures used for laboratory diagnosis (Smith et al., 1993). As an alternative to cell culture systems, ELISA is the first means of detecting viral antigens directly from extracts of clinical specimens.

For virus antigen detection, either the antibody sandwich or the competitive ELISA assay can be used (Hsiung, 1994). In the antibody sandwich method, a specific antibody to the antigen to be detected coats the surface of a solid-phase support, such as polystyrene beads, microtiter plates, or test tubes. Then the test sample (for example, stool, nasopharyngeal aspirate) is added and allowed to react. Detection of the antigen is then determined in two ways: either by the direct, single antibody sandwich assay, or by indirect, double antibody sandwich assay. For the direct or single antibody sandwich test, the enzyme conjugated to a specific antibody (recognizing a different epitope of the antigen) is then added and allowed to react. For the indirect or double antibody sandwich test, the unlabeled specific antibody is first added, then enzyme conjugated anti-globulin is added. As a final step, the amount of enzyme bound is detected by the addition of a substrate.

In earlier time, only polyclonal antisera produced in animals were available for the identification of virus isolates; variations in quality were problematic, and sensitivity as well as specificity varied. In recent years, MAb technology has greatly improved the assay in sensitivity and specificity. ELISA kits are now available commercially for many viruses including Measles, RSV, HIV, Rubella, Rota, CMV, VZV, HSV and Hepatitis B etc. (Hsiung, 1984).

However, most of these kits are not extensively or independently used for virus diagnosis due to their relative lack of sensitivity. In the diagnosis of HSV, commercial

available kits including Ortho ELISA and DuPont test kit are of low sensitivity in identifying HSV antigen from direct preparations or have to be supported by DNA analysis to confirm the detection and the type of HSV viruses in exposed patients (Gonik et al., 1991; Markoulatos et al., 1997). Another example is of antigen detection of HIV. Two commercial assays, Abbott HTLV III Antigen test and DuPont HIV p24 test, are currently used for detection of HIV viral antigen in clinical disgnosis. However, the low sensitivity of these methods severely hampers the practical value of p24 antigen as a virus load marker in anti-retroviral treatment monitoring (Piatak et al., 1993; Kappes et al., 1995).

Efforts have been made to improve the sensitivity of p24 antigen assay. Recently, a signal-amplification-boosted HIV-1 p24 antigen assay with heat-denatured plasma was developed (Boni et al., 1997). The presence of endogeneous human p24-specific antibodies, which complex the HIV antigen, causes under estimation of viruses in plasma. In this assay, heat denaturation prior to antigen testing destroys the antigen-binding capability and eliminates any human antibody interference. The mouse anti-p24 MAb and HRPO conjugated anti-mouse secondary antibody were used for detection via an indirect sandwich ELISA. HRPO was used to catalyze tyramide signal amplification (TSA) (Section 1.2.3.2). This method increased the sensitivity of p24 assay and the p24 antigen can be detected as low as 200 fg/ml.

1.5. Virus Purification

1.5.1. General Methods

Density gradient centrifugation (Brackke, M.K. 1951 and 1967), which was introduced 40 years ago, has been a widely used method for purifying virus particles and subviral components. However, this technique has several disadvantages (Schloer and Breese, 1982; Crooks et al., 1985). Firstly, density gradient centrifugation often causes loss of virus infectivity. For example, only 30% of infectivity was retained after $CsCl_2$

gradients in the case of equine herpes type 1 virus. As a result, these damaged virus particles were unsuitable for preparing samples for high-resolution techniques such as neutron scattering, X-ray scattering in solution and X-ray crystallography. Secondly, sophisticated, high speed machines capable of generating very high *g* values are required to generate $CsCl_2$ gradients for long periods of time. Instrument failure can pose a significant hazard when preparing pathologic viruses or subunits derived from them.

Several methods have been developed to overcome the problems associated with density gradient centrifugation, but each has it own limitation. Isoelectric focusing in nonionic detergent solutions has been used successfully for the purification of several viral proteins (Morein et al., 1978). However, this technique is only suitable for monomeric proteins which are almost invariably nonimmunogenic. Other methods including gel exclusion chromatography (Crooks et al., 1985), and ion-exchange chromatography lack specificity and cannot distinguish between viral proteins and cellular proteins with similar size and charge distributions. This is particularly important for enveloped viruses, as most of them have glycosylated surface antigens which would contain sugar residues similar to those found on cellular membrane glycoproteins.

1.6. Aims, Objectives and Hypothesis

A. Hypothesis

BsMAbs are unique immunoprobes incorporating two different paratopes in one molecule. Advantages of these molecules in immunoassay and immunodiagnosis have been recognized more then 15 years. We hypothesize that a bsMAb with one paratope specific to a virus and a second paratope to an enzyme could have high specificity and low background compared to the anti-virus polyclonal antibody or monospecific MAb. Exploiting this bsMAb probe and combining it with an amplified cyclic ELISA, we hope to develop a simple ultrasensitive method for virus detection and viral load estimation. Such a method, if developed, could be a viable alternative to complex and expensive PCR methods.

B. Aims:

The aims in this thesis were to develop a bsMAb with one paratope against phage M13 as a model virus and another paratope binding to a signal generating enzyme (such as AP and HRPO) and investigate the utilities of this new immunoprobe in three areas:

(1) development of an ultrasensitive ELISA for virus detection employing M13 phage as a model virus.

(2) development of a phage ELISA for rapid screening of a phage peptide library instead of traditional nonspecific plaque assay.

(3) development of bsMAb based affinity chromatography with a mild elution for recovery of intact viruses.

C. Specific Objectives:

(1) To develop a hybridoma secreting MAb against phage M13 coat protein.

(2) To develop a quadroma or trioma secreting bsMAb with one paratope binding to the phage M13 coat protein and second paratope to AP or HRPO.

(3) To purify the anti-phage M13/anti-AP bsMAbs free from parental anti-phage M13 monospecific MAbs using Mimetic blue A6XL affinity ligand, which has a high affinity for AP.

(4) To develop an ultrasensitive virus ELISA using an anti-phage M13/anti-AP bsMAb as the immunoprobe coupled with an enzyme amplified method.

(5) To develop a phage ELISA and establish a rapid and simple method for screening novel protein/peptide ligands from a phage display peptide library.

(6) To construct a bsMAb based affinity chromatography method for purification of viruses using M13 as a model virus.

Chapter 2. Production and Characterization of Monoclonal Antibodies Against Phage M13

2.1. Introduction

M13 phage belongs to a filamentous bacteriophage group which includes M13, f1 and fd. M13 contains single strand DNA in its genome and has a MW of approximately 1.2-1.4 x 10^7 Da. The phage coat consists of about 2700 copies of a major protein and five copies each of four minor proteins (Chapter 1.3.1). In this decade, filamentous phages have been used widely as cloning vectors to express peptide libraries or proteins fused to one of the phage coat or tail proteins. This phage display technology has been used for selecting peptides and proteins with desired properties, which have resulted in the discovery of new drugs and the development of novel proteins (Chapter 1.3.2). Since phage display technology has many potential applications, an anti-M13 MAb would be very useful in detecting the binding of phages displaying the desired peptide/protein with their target molecules that are used for selection.

Anti-M13 phage MAbs have been previously developed by several groups (Dente et al., 1994; and Bhardwaj et al., 1995). These MAbs proved very useful in detecting the binding of phages that displayed ligands fused to g3p (tail fibers) and were captured by immobilized receptors on a microtiter plate. I hypothesized that a bsMAb with one paratope bound to phage coat protein and the other paratope bound to a signal generating molecule such as AP would have a higher detection sensitivity with lower background than a conventional MAb used for the same purpose. To develop a quadroma secreting anti-phage M13/anti-AP bsMAbs, the first step was to prepare anti-phage M13 primary hybridomas. In this chapter, I describe the development and characterization of several anti-phage M13 primary hybridomas. The purified anti-phage MAbs are also needed as the solid phase capture antibodies in the subsequent development of phage sandwich ELISA.

2.2. Materials and Methods

2.2.1. Materials

Bacterial media components, streptomycin-penicillin-glutamine and fetal bovine serum (FBS) were obtained from Gibco BRL (Gaithersburg, MD, USA). Balb/c mice and the SP2/0 myeloma cell line were purchased from the University of Alberta and th eATTC (U.S.A.) respectively. Polyethylene glycol (PEG) 1300-1600, Freund's adjuvants, RPMI medium, OPI supplement, HAT supplement and goat anti-mouse IgG conjugated with HRPO (horseradish peroxidase) and goat anti-mouse (whole molecule) were acquired from Sigma Chemical Co. (St. Louis, MO, USA). Growth factor was from IGEN Inc. (Rockville, MD, USA). Reagents for sodium dodecyl sulfate-polyacylamide gel electrophoresis (SDS-PAGE) were from Bio-Rad (Rich-mond, CA, USA). PVDF membranes were purchased from Millipore (Bedford, MA, USA). The 96 well ELISA plate was from Nunc (Naperville, IL, USA). All other reagents and chemicals were of analytical grade. Wild typed bacteriophage M13 and the *E.coli* strain K91 were obtained from Dr. Bruce Malcolm, Department of Biochemistry, University of Alberta, Edmonton, Alberta. Fd phage displaying a biotin mimetic peptide was provided by Dr. F. Jacobs, Biomira Inc., Edmonton.

2.2.2. Development of Anti-phage M13 Hybridomas

2.2.2.1 Antigen Preparation

The wild type M13 bacteriophages were purified by a $CsCl_2$ gradient using the following procedure (Lin et al.,1980): (1) 10 mL of bacterial *E.coli* K91 was mixed with 200 µl of the M13 bacteriophage stock in a sterile tube and this tube was incubated for 20 min at RT; (2) the infected *E.coli* was inoculated into four 1-litre flasks (2.5 mL inoculates/flask) containing 250 mL fresh medium, and the flasks were incubated for 16 hrs at 37^0C with constant vigorous shaking; (3) the *E.coli* cultures were centrifuged at

5000 rpm (g=4420) for 20 min at RT in a Sorvall GS3 rotor; (4) the *E.coli* supernatants were re-centrifuged at 8000 rpm (g=11300) for 10 min; (5) PEG 8000 (39 mL of 20% PEG/2.5 M NaCl filtered stock solution per 310 mL phage suspension) was added to *E.coli* supernatant to precipitate the phages and these solutions were mixed thoroughly and stored at 4°C overnight; (6) phages were collected by centrifugation at 8000 rpm for 40 min at RT; (7) the supernatants were removed completely (invert, then aspirate) from phage pellets and the pellets was resuspend in 15 mL 1 x TBS; (8) the resuspended phages was poured into a plastic beaker and 1 x TBS was added to get a final net weight of 18.99g; (9) 8.53g $CsCl_2$ was added to phage solution and mixed by swirling to make a final volume of ~21 mL with a density of 1.30g/mL; (10) the phage-$CsCl_2$ solution was added to SW41 polyallomer tubes and the tubes firstly were filled close to their rim, then put into the SW41 swing buckets, then filled completely, then balanced in the SW41 swing buckets with the SW41 caps; (11) the phages were centrifuged at 37,000 rpm (g=22803g) for 48 hr at 15°C using Beckman ultracentrifuge; (12) the phages were removed from tubes with a syringe and 18 gauge needle (the phage appeared as a broad, faintly bluish band); (13) the phage sample was dialyzed against 4 L PBS for 24 hr with three buffer changes.

$CsCl_2$ gradient purified phages were titered by plaque assay as following:

The phage solution was diluted by a logarithmic series in LB medium (bacto-tryptone 10g/L, bacto-yeast extract 5g/L, NaCl 10g/L). A 100 µl volume from each dilution was mixed with 100 µl of fresh *E.coli* K91 growing in log phase (O.D.=0.6-0.8) in a sterile tube and vortexed gently. After adding 3 mL LB top agar medium (LB medium plus bacto-agar 7g/L) into the tube and gently vortexing, the contents of this tube were immediately poured onto a plate containing 30-35 mL of hardened LB bottom agar medium (LB medium plus bacto-agar as 15g/L). After the plate was incubated 10 hr at the 37°C, plaques were counted.

The purity of $CsCl_2$ purified M13 phages were checked by SDS-PAGE and silver staining as follows:

The $CsCl_2$ purified M13 phages were boiled in 2% SDS sample buffer for 5 min. Approximately 10^{10} pfu phages were then loaded into each lane on a 0.4% SDS/12.5%

polyacrylamide gel. After electrophoresis in a miniprotein II apparatus (BioRad) for 1 hr at 150 V, the gel was stained by the diamine silver staining method (Nauta and Gygax, 1951).

2.2.2.2. Immunization

Three balb/c mice were immunized with $CsCl_2$ purified M13 phages following an immunization protocol described in Table 2.1. After the third immunization by intraperitoneal injection, the mouse were bled by the tail vein and the titer of anti-phage M13 antibodies in serum was determined by a direct binding ELISA (Fig.2.1A) as follows:

A 96 well microtiter plate was coated with $CsCl_2$ purified M13 phages at 10^{10} pfu/well overnight at 4^0C. The plate was washed and incubated with 5% skim milk in PBS to block non-specific binding sites. The plate was washed three times with PBS-T (PBS, pH 7.2, containing 0.1% Tween 20). A 100 µl volume of mouse serum at 1:1000 dilution was added to individual wells and incubated for 1 hr at RT. After washing, 100 µl of goat anti-mouse IgG HRPO conjugate at 1:1000 dilution was added and incubated for another 1 hr. Following the last washing step, the binding of anti-phage antibodies was determined by adding 100 µl of 0.3 mg/mL ABTS (2'-azino-bis, 3-ethybenzthiazoline-6-sulfonic acid) containing 0.03 % H_2O_2. The plate was incubated for 10 min and an optical density at 405 nm was measured using a plate reader (Molecular Devices, USA).

2.2.2.3. Fusion of cells for producing anti-phage M13 hybridomas

The anti-phage M13 hybridomas were produced by fusing the spleen cells from immunized mice with SP2/0 myeloma cells following the fusion procedures below: (1) the spleen was excised from the immunized mouse on the third day after the intrasplenic injection, then the excised spleen was flushed with 10 mL warm serum free medium using 5 mL syringe with a 26 gauge needle and the spleen cells were collected and

Table 2.1. Immunization protocol

Time (days)	Amount of Ag[a]	Adjuvant	Volume	Route
1	~50μg (10^{11} Phages)	FCA[b]	0.2 mL	IP[c]
8	~50μg (10^{11}phages)	FICA[d]	0.2 mL	IP
10	~100μg (10^{12} phages)	PBS[e]	0.5 mL	IP
12	~50μg (10^{11}phages)	PBS	0.2 mL	IS[f]
15	Fusion			

a = Purified bacteriophage M13, b = Freund's complete adjuvant,

c = Intraperitoneal injection, d = Freund's incomplete adjuvant,

e = Phosphate buffer saline, f =Intrasplenic injection.

counted. (2) the spleen cells were mixed with SP2/0 cells in a 5:1 ratio and the mixed cells were washed three times with 20 mL serum free RPMI-1640 medium. (3) the medium was aspirated out from the pellet after the final wash as much as possible, then 0.5 mL prewarmed PEG (pH 8.0) was added to the cell pellet drop by drop for 45 sec to 1 min with gentle shaking and the cells were centrifuged for 7 min at 200 g. (4) the cells were resuspend in 5 mL serum free RPMI-1640 medium slowly. (5) 5 mL RPMI-1640 medium containing 20% FBS was added slowly to cell suspension with gently shaking, then the cells were centrifuged for 5 min at 200 g and the supernatant was taken out. (6) the cells were washed with 20% FBS RPMI-1640 medium 3 times and resuspend at a density of 10^6 cells/mL in same medium supplemented with 2 mM L-glutamine, 50 U/mL penicillin and streptomycin, 20% v/v of FBS, 10% Origen, OPI and HAT. (7) a 200 µl aliquot of cell suspension was seeded into each well of 96-well cell- culture plates and cells were incubated at 37^0C, in a humidified incubator with a 5% atmosphere.

2.2.2.4. Screening for anti-phage M13 hybridomas

Screening for positive hybridomas started about the fourteenth day after fusion using a direct binding ELISA as described in section 2.2.2.2 (Fig.2.1A). A 100 µl aliquot of cell culture supernatant collected from different clones was added to $CsCl_2$ purified phage coated plate for ELISA analysis (Screen 1). The positive primary hybridoma clones were transferred to 24-well culture plates followed by 6 well culture plates and ELISAs were performed using 24-well culture supernatant (Screen 2) as well as 6-well supernatant (Screen 3) for keep testing the presence of anti-phage MAbs. The positive clones were frozen and stored in liquid nitrogen.

2.2.2.5. Cloning and recloning of selected hybridomas

In order to obtain monoclonal cultures, the 5 selected hybridoma clones were selected by limiting dilution. The primary hybridomas growing in 6-well culture plate were counted to determine the cell concentration. The cells were diluted in series to

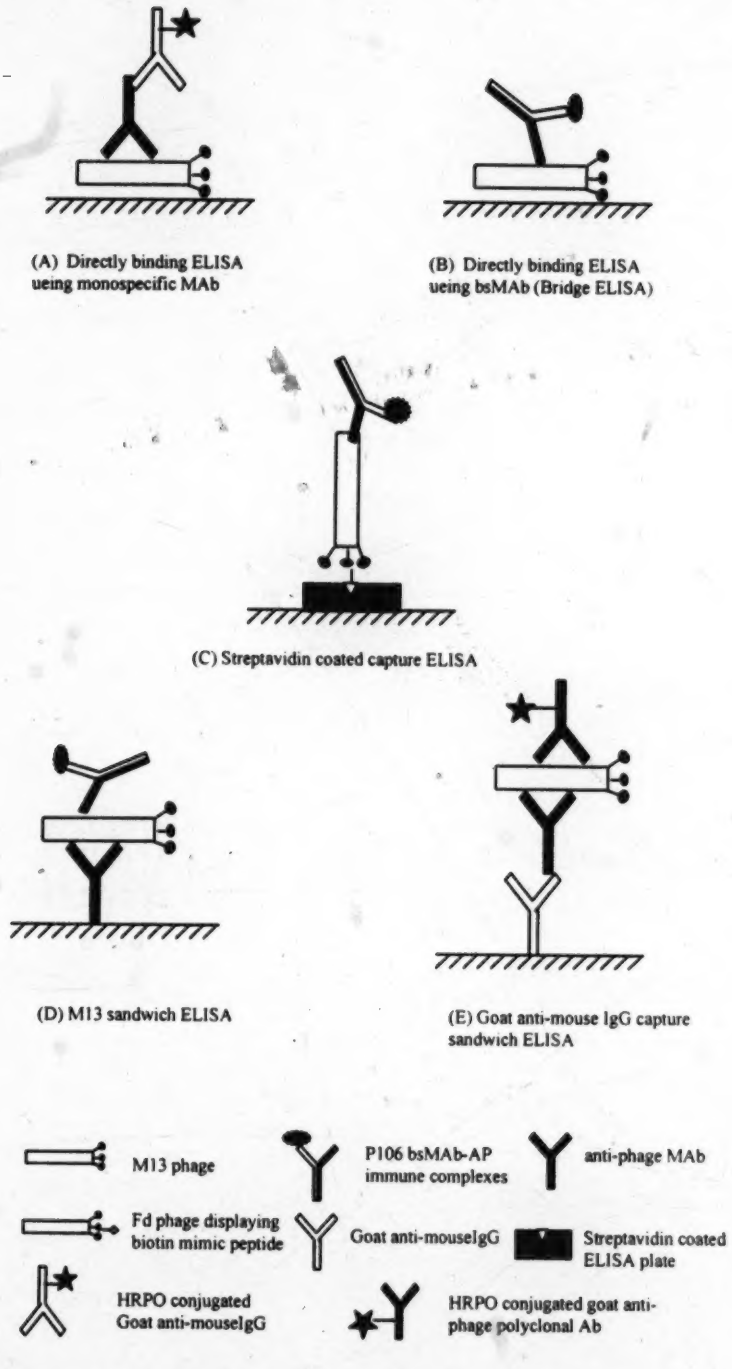

(A) Directly binding ELISA ueing monospecific MAb
(B) Directly binding ELISA ueing bsMAb (Bridge ELISA)
(C) Streptavidin coated capture ELISA
(D) M13 sandwich ELISA
(E) Goat anti-mouse IgG capture sandwich ELISA
M13 phage
P106 bsMAb-AP immune complexes
anti-phage MAb
Fd phage displaying biotin mimic peptide
Goat anti-mouseIgG
Streptavidin coated ELISA plate
HRPO conjugated Goat anti-mouseIgG
HRPO conjugated goat anti-phage polyclonal Ab

Fig.2.1 Schematic representation of ELISA formats used in this thesis.

obtain a final concentration of 5 cells/mL. A 100 µl aliquot of cell suspension was added to each well of 96-well plates and the plates were incubated in a CO_2 incubator at 37^0C. After 14 days, the hybridomas were rescreened for M13 binding activities by ELISA. The limiting dilution was repeated until a 100% percent positive hybridoma was achieved in the culture plate.

2.2.3. Characterization of Anti-phage M13 Monospecific MAbs

2.2.3.1. Antibody isotyping

The isotypes of anti-phage M13 MAbs were determined using the Isostrip™ kit (Boehringer Mannheim, Germany) following the manufacturer's instructions. The culture supernatant of the test MAb was diluted 1: 100 in PBS pH 7.2-7.6. A 150 ul aliquot of diluted sample was added to the developmental tube and the tube was incubated at room temperature for 30 seconds follow by agitation briefly so that colored latex was completely resuspended. The isostrip was placed in the developmental tube with black end of the isostrip at the bottom. Within 1-5 minutes, a blue band appeared in one of sections coated with goat anti-mouse antibodies against different subclasses and the location of blue band indicated the subclass of a MAb.

2.2.3.2. Western blotting for determining specificity of MAbs against phage coat proteins

$CsCl_2$ purified M13 phages were boiled in 2% SDS sample buffer for 5 min Approximately 10^{10} pfu phages were subjected to electrophoresis in 12% SDS-PAGE using miniprotein II apparatus (BioRad). After electrophoresis, phage proteins were transferred to a PVDF membrane. The membrane was cut into strips and was probed individually with anti-M13 MAb from different cell culture supernatants. The membrane strips were washed three times with PBS-T and incubated with HRPO-conjugated goat anti-mouse IgG for 2 hr. Following a final washing step, the binding of anti-phage M13

MAbs to phage coat proteins were detected by the blue bands of insoluble product wherein the strips were incubated with 1 mg/mL 4CN (4-chloro-1-napthol) in PBS containing 0.09% H_2O_2. The reaction was stopped by washing with tap water.

2.2.3.3. Relative avidity analysis of anti-phage M13 hybridomas

Four anti-phage M13 hybridomas were analyzed for relative MAb avidity against M13 phage using a goat anti-mouse IgG capture sandwich ELISA (Fig.2.1E). To normalize the different culture supernatants secreting varied amounts of MAbs, the wells were coated with a limited amount of solid phase capture antibody (0.1 μg/well goat anti-mouse). The plate was blocked with 5% skim milk in PBS followed by washing (three times) with PBS-T. A 100 μl aliquot of cell culture supernatants collected from different clones was added in triplicate to the wells and the plate was incubated for 1 hr at RT. It was assumed that about the same amount of mouse MAbs were captured by a limited amount of coating antibodies in each well during the incubation since MAbs in culture supernatant were in vast excess compared with coating antibodies. The plate was then washed 3 times with PBS-T and 100 μl of M13 phages in a log serial dilution were added. The bound phages were detected by polyclonal anti-phage M13 antibody HRPO conjugates and signal development was the same as in section 2.2.2.2.

2.2.3.4. Crossreactivity with fd phage

Crossreactivity of anti-M13 phage with fd phage was determined by a streptavidin capture ELISA (Fig.2.1C). The ELISA plate was coated with 100 μl of streptavidin (10 μg/mL in PBS) overnight at 4°C. The plate was blocked with 3% dialyzed BSA for 2 hr at RT followed by washing with PBS-T three times. Approximately 10^{10} pfu fd phages displaying biotin mimic peptide were added to the wells and incubated for 1 hr at RT. After washing, 100 μl of culture supernatants from different hybridoma clones (total 40 clones) were added to individual wells and incubated for 1 hr followed by another washing. A 100 μl aliquot of HRPO-conjugated goat anti-mouse IgG was added and

incubated for 1 hr to detect the binding between anti-phage M13 MAbs and fd phages. The color development was the same as described in section 2.2.2.2.

2.2.3.5. C[illegible]eactivity with E.coli proteins

E.coli K91 was cultured in 10 mL of LB medium overnight at 37°C with constant shaking. On the following day, the cells were was harvested, resuspended in 2 mL PBS and sonicated. The ELISA plate was coated with 100 µl of soni[illegible]ted *E.coli* protein solution overnight at 4°C. After blocking with 5% milk for 2 hr at RT and washing with PBST, 100 µl of cell culture supernatants collected from different clones (total 40 clones) were added to the plates and incubated for 1 hr at RT. Following a washing step, then 100 µl of HRPO-conjugated goat anti-mouse IgG were added to each well and incubated for another 1 hr to detect any MAbs which were binding to *E.coli* proteins in the wells. The color development was the same as described in section 2.2.2.2.

Five selected anti-phage M13 clones, P93.3, P93.5, P93.8, P93.11 and P93.13, were also checked for their crossreactivity with *E.coli* proteins by Western blot analysis. About 100 µg of sonicated *E.coli* extract in 60 µl SDS sample buffer was loaded into each lane of the 2% SDS-PAGE gel. The Western blot was performed following the same procedure as described in section 2.2.3.2.

2.2.4. Purification of Anti-phage MAbs

2.2.4.1. Ascites production

The best anti-phage M13 producing hybridoma clone (P93.11) was chosen for ascites production based on its high avidity against phage M13. About 2.5 x 10^6 hybridoma cells were suspended in 1 mL PBS and this cell suspension was intraperitonealy injected into a balb/c mouse after one week of Pristane injection. Ascites was collected after 10 days and centrifuged for 15 min at 3000 rpm (g=1090) to recipitate cells and lipid.

2.2.4.2. Purification of MAbs

Purification of P93.11 was achieved by ammonium sulphate precipitation followed by ion exchange chromatography. Ammonium sulphate was gradually added to ascites with stirring to achieve 50% saturation and the solution was left overnight at 4^0C. The following day, the precipitate was collected by centrifuging for 30 min at 3000 rpm. The pellet was dissolved in 20 mL of 10 mM Tris-Cl, pH 8.6 and dialyzed exhaustively with five changes of 2000 volumes of 10 mM Tris buffer. The sample was loaded on to a 15 mL DE-52 column at a flow rate of 1 mL/min. The column was washed with Tris buffer until all the unbound materials were removed as determined by UV monitoring at A_{280} with a UV monitor. Bound Abs were eluted by a linear gradient of 0-to 0.5 M NaCl in 200 mL Tris buffer at a flow rate of 0.5 mL/min. Fractions were collected and checked for MAb activity by ELISA. Fractions with the highest activity were pooled, and the purity of the MAb was determined by SDS-PAGE.

2.3. Results and Discussion

2.3.1. Development of Anti-phage M13 Hybridoma

In order to develop an anti-phage M13 hybridoma, the first step was to prepare enough antigen to stimulate a good antibody response in immunized mice. In the first fusion experiment, the results show (Table 2.2) that most antibodies elicited were anti-*E.coli* proteins since partially purified phage (PEG precipitation) was used to immunize mice. This can be explained by the fact that *E.coli* proteins are likely stronger immunogens than phage proteins. In this situation, screening for the desired hybridoma was difficult unless pure phage proteins were prepared for screening. It was considered that the development of anti-M13 hybridomas could be improved by using highly purified phage particles as immunogens. Highly purified M13 phages were obtained by $CsCl_2$ gradient purification (Lin, 1980) in good yields from a bulk *E.coli* culture infected with M13.

Antigen coated on ELISA plate	Group 1 mouse serum[a]	Group 2 mouse serum[b]
$CsCl_2$ purified phage particles	not tested	+++
PEG purified phage particles	++++	++++
Sonicated *E.coli* proteins	++++	+

a. Serum from the mouse immunized with PEG purified phage particles.

b. Serum from the mouse immunized with $CsCl_2$ gradient purified phage

Table 2.2 Serum response to PEG and $CsCl_2$ gradient purified M13 phage: Two separate groups of mice (3 for each group) were immunized with either PEG purified phage particles or $CsCl_2$ gradient purified phage particles. On the third day after the third immunization, the mice were bled by tail vein and sera were checked by a direct binding ELISA using $CsCl_2$ purified phage particles or PEG purified phage particles or sonicated *E.coli* proteins as the solid phase antigen.

The purity of the phage sample was checked by SDS-PAGE and silver staining. The results shown in Fig.2.2 indicate that most *E.coli* proteins were removed from the $CsCl_2$ gradient purified M13 phage sample and as just two protein bands were seen: namely the phage tail protein (56Kd) and the phage major coat protein (14Kd). In contrast, the PEG-purified phage precipitate showed some contaminating bands likely derived from *E.coli*. The highly purified phage particles were used for immunizing mice following a protocol described in Table 2.1. The mouse serum was analyzed by ELISA before fusion. The ELISA results show (Table 2.2) that all three mouse sera immunized with $CsCl_2$ purified phages had high antibody activity against $CsCl_2$ purified phages and only very low crossreactivity with *E.coli* proteins using a direct binding ELISA. The splenocytes from immunized mice were fused with SP2/0 myelomas using polyethylene glycol (PEG) following a protocol described in section 2.2.2.3. Cells were selected in HAT medium based on the fact that the myeloma ($HGPRT^-$ cells) can't grow in HAT medium (referring to Chapter 1.3), and the unfused or fused spleen cells had only a short life *in vitro* since they were untransformed cells. Only hybrid cells (myeloma x B cells) were capable of surviving in HAT medium.

The screen for anti-phage hybridomas was performed on the 14th day after fusion by direct binding ELISA of $CsCl_2$ purified phage-coated microtitre plates. Since hundreds of hybridoma clones showed positive ELISA in the first screen using 96-well culture supernatants, a value of optical density (O.D.) above 0.7 was chosen as cut off value for selecting primary hybridomas. In total, 60 primary hybridoma clones were selected with an O.D. higher than 0.7 above background. The higher ELISA signal in this assay was not necessarily indicated high antibody affinity since each tested supernatant contained different amount of MAbs. Second and third screen were performed using cell supernatant from 24 well and 6 well of each cultured clone. All positive primary clones after the third screen were frozen in liquid nitrogen.The data from the primary screen experiment is shown in Table 2.3.

Recloning is required to avoid polyspecificity of the culture which would also assume the risk of causing nonproducing cells to overgrow. It was observed in my screening experiment some primary positive clones lost antibody activity during the 2

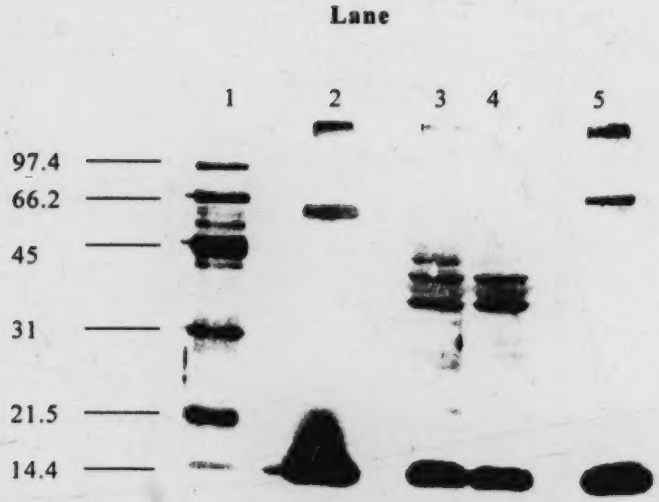


Fig. 2.2 SDS-PAGE and silver staining of phage M13 coat proteins. Approximately 10^{10} M13 phage were boiled in 2 % SDS sample buffer for 5 min and electrophoresed on 12% polyacrylamide gel. Proteins were visualized by silver staining. Lane 1, LMW marker; lane 2 and lane 5, $CsCl_2$ gradient purified phage M13 show a massive lower molecular weight coat protein at 14 Kd and a tail fiber at 57 Kd; lane 3 and lane 4, PEG purified phage M13 exhibiting considerable impurities putatively derived from *E.coli.*

Clones	Screen 1 (O. D.)	Screen 2 (O. D.)	Screen 3 (O. D.)	Clones	Screen 1 (O. D.)	Screen 3 (O. D.)	Screen 4 (O. D.)
1-1	2.960	2.356	1.250	11-8	1.205	1.036	0.985
1-13	4.000	3.582	3.695	11-9	2.539	2.356	2.134
1-19	0.395	0.205	0.056	11-10 (P93.11)	2.892	2.742	2.354
1-27	2.510	2.584	1.985	12-14	2.387	2.214	1.987
2-27	1.809	1.052	0.862	13-1	1.888	1.759	1.768
2-6	1.306	1.258	1.020	13-5	2.250	2.031	1.954
2-27	1.809	1.985	1.896	13-10	1.264	1.132	0.841
2-36	1.237	1.135	1.023	13-11 (P93.13)	2.023	2.013	1.978
3-3 (P93.3)	0.879	0.825	0.532	13-22	1.243	1.132	0.742
3-17	2.053	1.092	1.530	14-1	2.114	2.124	0.765
3-30	2.884	2.456	2.005	14-3	1.060	0.562	0.325
3-25	3.596	3.214	3.502	14-6	2.631	2.345	1.852
3-15	2.053	2.250	1.962	14-12	1.417	1.224	0.968
3-11	2.656	1.230	0.563	14-15	1.747	1.454	1.258
4-29	0.932	0.423	0.103	14-17	2.352	2.225	2.034
5-3 (P93.5)	3.297	2.986	3.021	14-26	2.274	2.036	1.854
5-4	1.316	1.254	1.236	14-27	1.483	1.235	1.204
7-7	1.825	1.257	0.986	14-28	1.966	1.582	1.462
8-8 (P93.8)	3.058	3.245	3.261	14-4	4.000	3.896	3.758
8-3	1.113	1.023	0.968	14-12-1	3.474	3.252	3.045
8-20	2.135	2.123	1.958	14-19	2.957	2.458	2.145
10-5	3.101	2.561	2.043	20-9	3.058	2.457	2.058
10-7	1.879	1.563	1.258	20-4	2.510	2.135	2.041
11-7	1.495	0.965	0.324	20-23	0.804	0.654	0.568
11-4	0.879	0.584	0.123	20-25	1.574	1.896	0.452
11-6	1.327	1.369	1.254	20-15	2.512	2.385	2.014

Table 2.3 ELISA for screening anti-phage M13 primary hybridoma clones: On about the fourteenth day after hybridoma fusion, the supernatants from different clones were checked by a direct binding ELISA of $CsCl_2$ gradient purified phage-coated plates. The binding of MAbs was detected (screen 1) by HRPO conjugated goat anti-mouse IgG (whole molecule). The positive clones were retested after transfered to 24 well plate (screen 2) and 6 well plate (screen 3). The maximum optical density is 4.000 for this assay.

week culture period. Five clones (P93.3, P93.5, P93.8, P93.11, and P93.13) were selected for recloning by limiting dilution based on their isotype and initial high reactivity against M13 phage.

2.3.2. Characterizations of anti-phage MAbs

2.3.2.1. Isotype of anti-phage M13 MAbs

The most common type of MAbs is the monospecific bivalent form belonging to the IgG class of immunoglobulin. Monoclonals of the IgM class as well as the other much less frequent IgA, IgD and IgE classes have also been reported (Suresh, 1986a). The IgG class is the most commonly used isotype for developing bsMAbs. IgM isotypes with stronger avidity have advantages in being used as the capture antibodies. Five hybridoma clones were checked by the commercial Isostrio™ kit where the strips were immobilized with goat anti-mouse antibodies against different subclasses. The results shown in Table 2.4 demonstrate that the four clones (P93.3, P93.5, P93.8 P93.11) were of the IgG isotype and one (P93.13) was of the IgM isotype.

2.3.2.2. Specificity of MAbs against phage coat proteins

To determine the specificity of the developed MAbs against either the phages major coat protein or tail fibers, cell culture supernatants from 40 different clones were subjected to Western blot analysis. Eight of 40 clones checked were shown to have specificity against M13 major coat proteins, which bound and identified the 14 Kd band on nitrocellulose. Two of them are P93.3 and P93.13. (Fig.2.3). The rest of the 32 clones didn't bind either the major coat proteins or the tail fibers on the blot. One explanation for 'western negative' clones could be due to the reactivity of the MAbs to conformationally sensitive epitopes of the M13 coat protein . The major coat protein has only 50 amino acids (Henry,1969) and may have few exposed epitopes. Some of these are altered by SDS and the Western blot procedure. The eight clones mentioned above were able to

Clones	Isotype	Reactivity with fd phage	Reactivity with phage major coat proteins on western blot	crossreactivity with *E.coli* proteins
P93.11	Ig2a	++++	-	-
P93.8	Ig2a	++++	-	-
P93.3	IgG1	++++	++	-
P93.5	Ig2a	++++	-	-
P93.13	IgM	++++	++	-

Table 2.4 Summary of characteristics of five selected primary hybridoma clones. The isotypes of five Ab secreting hybridoma clones supernatants were determined by the commercial isotype strips immobilized with goat anti-mouse antibodies against different subclasses. Their reactivity with fd phage was checked by a streptavidin capture ELISA wherein streptavidin on a solid phase captured fd phage displaying biotin mimetic peptide and captured fd phage could be detected by anti-phage M13 MAbs plus goat anti-mouse HRPO conjugate. Western blot was performed to determine the specificity of each clone supernatant against phage coat proteins. Crossreactivity of these clones with *E.coli* proteins were tested by a direct binding ELISA of sonicated *E.coli* proteins.

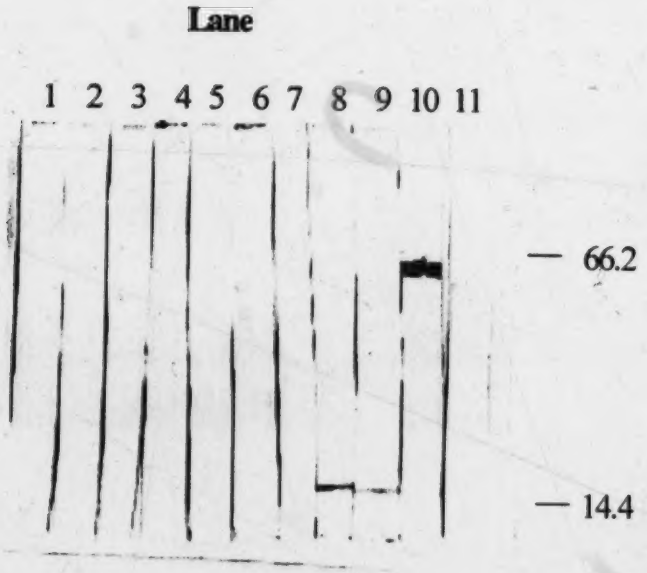


Fig. 2.3 Western blot analysis for determining the specificity of anti-phage M13 primary hybridomas against phage coat proteins. About 10^{10} pfu of phage M13 particles were electrophoresed in 12% SDS polyacrylamide gel. and phage proteins were transferred to PVDF membrane. The membrane was cut into strips and probed individually with culture supernatants from different hybridoma clones. Lane 11, LMW marker; lane 10, stripe was probed with anti-phage M13 polyclonal Ab HRPO-conjugate; lanes 9 and 8, strips were probed with hybridoma clone P93.3 and P93.13 respectively, lane 7 to lane 1, strips were probed with hybidoma clones P93.11, P93.8, P93.5, P93.4, P93.3, P93.2 and P93.1.

detect M13 coat protein epitopes both in the native virus and in the denatured form on the Western blot. In addition, clones reacting with M13 tail protein were not generated in this fusion. This is possibly because phage M13 consist of ~ 2700 copies of the major coat proteins with only few fibers and other minor proteins (Henry,1969). It is interesting to note that on a relative scale, the polyclonal anti-M13 antibodies showed stronger reactivity with the tail fibers than the coat protein (Lane 10 of Fig.2.3). This could sterically inhibit the binding of the phage in a panning experiment or exhibit reduced binding to phage already bound to antigen on the solid phase.

2.3.2.3. Relative avidity analysis of anti-phage MAbs

In order to select the best anti-phage M13 secreting hybridoma for the development of a quadroma, four IgG clones described in section 2.3.2.1 were further analyzed by ELISA for their relative avidity against the M13 phage. Since measurement of absolute antibody affinity is complex and requires either purified monomeric coat protein antigens or purified MAbs or their fragments, the relative avidity of the four MAbs was compared using cell supernatant collected from each hybridoma clone. Anti-phage MAbs in supernatant were far in excess than solid phase capture antibodies. In this assay, a limited amount of goat anti-mouse IgG was coated in the ELISA plate wells so that each well was likely to capture an equal amount of mouse anti-phage MAbs from different hybridoma supernatants. M13 phages at various concentrations were added and incubated with MAbs immobilized on a solid phase by goat anti-mouse IgG. The captured phages were detected by anti-phage polyclonal antibody HRPO-conjugates. The MAb that showed a higher optical density at a lower phage concentration likely has a relatively higher avidity. As shown in Fig.2.4, P93.11 and P93.8 have a higher avidity against phage M13. Thus, hybridoma P93.11 and P93.8 were used in subsequent studies.

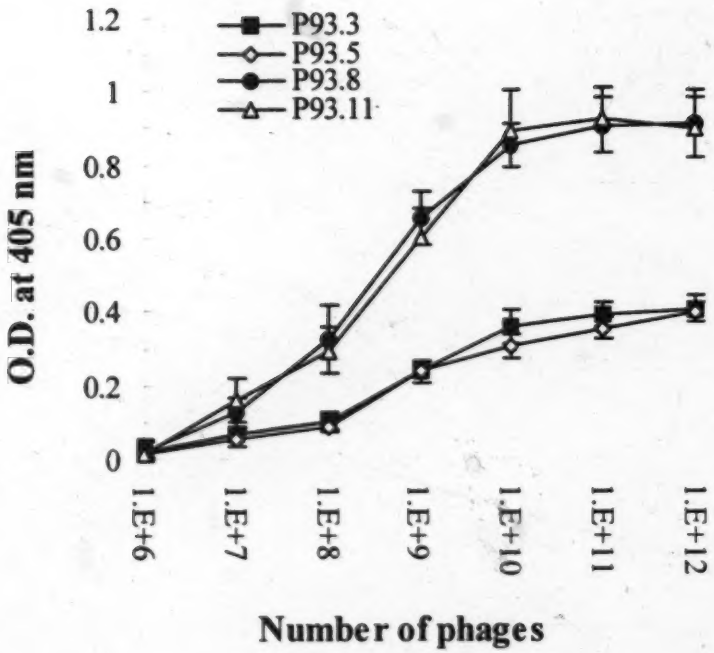


Fig. 2.4 Relative avidity analysis of different anti-M13 MAb clones: microtitre plate was coated with a limited amount of goat anti-mouse IgG. A 100 µl volume of cell culture supernatants from four individual clones was added to the plate followed by a 2 hr incubation and washed. A 100 µl volume of phages (10^{6}-10^{12}) was added into each well, incubated 2 hr and washed. The bound phages were detected by anti-phage polyclonal Ab HRPO conjugates. Each point represents a mean of triplicate. The MAb that shows higher optical density at lower phage concentration likely has a relatively higher avidity. As shown in this Fig., P93.11 and P93.8 have higher avidity against phage M13. Thus, hybridoma P93.11 and P93.8 were used in subsequent studies.

2.3.2.4. Cross-reactivity of anti-phage M13 MAbs with E.coli proteins

Although highly purified phages were used for immunization and screening, there would likely be some contaminating of *E.coli* proteins in the phage sample. These *E.coli* proteins could be stronger immunogens in stimulating an antibody response. It was, therefore, necessary to check whether the individually selected anti-M13 MAb bound *E.coli* proteins in a cross-reactivity experiment. For this purpose, *E.coli* cells was sonicated to release cytoplasmic proteins into solution and this sonicated *E.coli* sample was used for coating an ELISA plate. Cell culture supernatants from 40 different MAb clones were added for incubation with *E.coli* proteins and HRPO conjugated goat anti-mouse IgG was used for detecting the binding (cross-reactivity, if any) between anti-phage M13 MAbs and *E.coli* proteins on the ELISA plate. Five clones were further checked by western blot analysis. None of the clones, either by direct binding ELISA or western blot, showed any crossreactivity with *E.coli* proteins. These results are shown in Table 2.4.

2.3.2.5. Crossreactivity of anti-phage M13 MAbs with fd phage

Since fd phage has also been used widely in phage display technology, it was of interest to test whether our anti-phage M13 MAbs were also able to react with fd phages. An ELISA was performed on a streptavidin coated plate to capture fd phages displaying a biotin mimic on their tail proteins from an *E.coli* culture supernatant. The figure 2.1C schematically represents this streptavidin coated capture ELISA. *E.coli* protein contaminants can be removed by a wash step. Cell culture supernatants from different hybridoma clones were added to react with bound fd phage. Results indicate that anti-phage M13 MAbs bound fd phage with comparable strength to M13 phages. This is expected since fd, f1 and M13 are a group of related viruses and their coat proteins are almost identical (Chapter 1.7). The cross-reaction of anti-phage M13 MAbs with f1 phage was also shown previously (Dente et al., 1994). These results suggested that anti-phage M13 MAbs could be used for detecting many filamentous phage based vectors. In

addition, these results that anti-phage M13 MAb can detect fd phage further proved that these MAbs were specific to phage protein and not the *E.coli* contaminants. Table 2.4 summarizes all characteristics of the five selected hybridoma clones.

2.3.3. Purification of Anti-phage M13 MAbs

In order to develop a highly sensitive sandwich ELISA for detecting M13 phage in subsequent studies, pure anti-phage MAbs were required as the solid phase capture antibody. Although IgM MAbs can be used as efficient capture molecules, they are not easily purified by standard methods. Purification of IgG MAbs is somewhat easier. DE-52 ion-exchange chromatography was employed for P93.11 MAb purification. The purification profile is shown in Fig.2.5 wherein fractions 17, 18 and 19 represent the highest antibody activity detected by ELISA. These three fractions were pooled (6 mL) and their concentration was estimated to be 1 mg/mL by a Bio-Rad protein assay. The purity of the DE-52 fractions was checked by SDS-PAGE. The results in Fig.3.4 (next chapter) show that most contaminating proteins were effectively removed.

General methods for purifying antibodies include DE-52 ion-exchange chromatography and protein A/protein G affinity chromatography. DE-52 ion-exchange method is a low cost method and MAbs can be easily prepared. Although this method can lack specificity for a given antibody, the purified P93.11 MAbs proved to be very clean and hence was specifically used in this study. The protein A or protein G affinity method is a more specific and efficient method of separating antibodies from contaminants, however it is relatively expensive. More importantly, a relatively harsh elution condition (pH 2-3) has to be used, which might cause antibody denaturation.

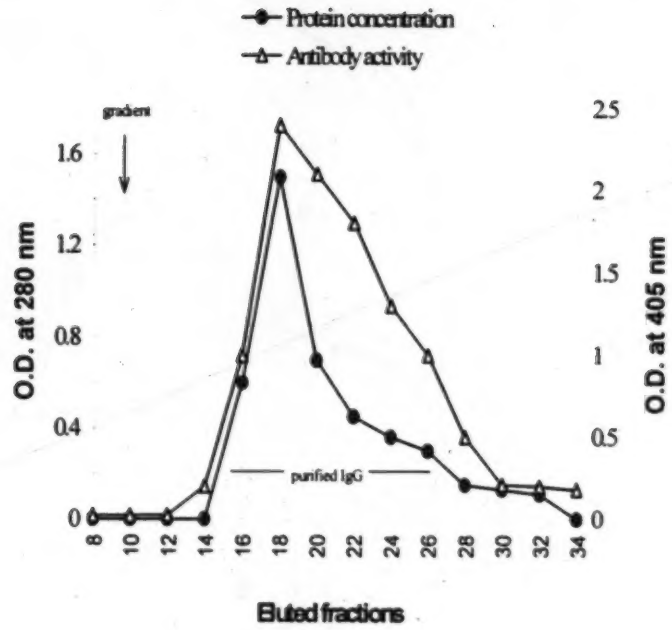


Fig. 2.5 DE-52 ion exchange chromatography purification of P93.11 MAb. Mice ascites were firstly precipitated by ammonium sulfate. The precipitates were dissolved in 10 mL of mM Tris buffer and dialyzed with 5 time changes of 2000 mL of 10 mM Tris buffer. The sample was load to column at a flow rate of 1 mL/min. After washing the column, the bound antibodies were eluted with a linear gradient of 0-0.5 M NaCl in 200 mL Tris buffer at a flow rate of 0.5 mL/min.. The fractions were collected and checked for both antibody activity and protein concentration.

Chapter 3. Production and Characterization of Bispecific Monoclonal Antibody

3.1. Introduction

A bispecific monoclonal antibody (bsMAb) is an antibody that has two different predetermined paratopes, one on each Fab arm. This novel design has many advantages over chemically conjugated monospecific monoclonal antibodies (MAbs). By employing a bsMAb as the immunoprobe, ELISAs have been greatly improved in sensitivity, reproducibility and assay linearity (Chapter 1.1.4). My main objective in this thesis was to develop and characterize an anti-phage M13/anti-AP bsMAb immunoprobe and investigate the applications of this new probe in virus detection and purification and in phage displaying peptide library screening.

The most commonly used method for the production of bsMAbs is the hybrid-hybridoma fusion technique. There are two types of hybrid-hybridomas commonly produced, namely, the trioma and the quadroma. The trioma is a fusion product of a hybridoma cell with spleen cell from the immunized mouse, while the quadroma is derived by fusing two well established hybridomas (Chapter1.1.2.2). In this chapter, I describe the production of an anti-phage M13/anti-alkaline phosphatase (AP) quadroma and an anti-phage M13/anti-horseradish peroxidase (HRPO) trioma. For the production of the quadroma, the method of electrofusion was used to fuse the two hybridoma cells. They were labeled with two different fluorescent dyes, and the fused cells were selected by a fluorescence activated cell sorter (FACS). For the production of a trioma, the PEG-mediated cell fusion was performed using splenocytes from the M13 phage immunized mouse and an anti-HRPO hybridoma.

Random recombination of the heavy and light chains synthesized by a hybrid hybridoma could lead to ten possible immunoglobulin forms. BsMAbs have to be purified from monospecific contaminants in order to acheive a maximum specific activity (Chapter 1.1.3.). In this chapter, anti-M13 phage/anti-AP bsMAbs were purified from

anti-M13 MAbs by a novel affinity method using Mimetic Blue A6XL for subsequent bsMAb applications

3.2. Methods and Materials

3.2.1. Materials

Fluorescein isothiocyanate (FITC), tetramethylrhodamine isothiocyanate (TRITC), pNpp (para-nitrophenyl phosphate) and ABTS (2.2'-azino-di[3-ethyl-benzthiazoline]) were purchased from Sigma. A fluorescence activated cell sorter (FACS III, Becton-Dickinson, Sunnyvale, CA) with an argon ion laser (Model 160-05, Spectra-Physics) was part of a centralized facility, Medical Microbiology Immunology Department in University of Alberta. The Electro cell manipulator 200 was from BTX, SanDiego, U.S.A.

3.2.2. Hybridoma Cell Lines

Hybridoma cell lines P93.11 (IgG2a) and P93.8 (IgG2a) secreting anti-M13 MAbs were produced and characterized as described in Chapter 2. The Hybridoma cell line P92, developed previously in our lab, was a mouse hybridoma secreting an anti-alkaline phosphatase IgG1 MAb (Xu et al., 1998). This cell line was well established and ready to be used as a fusion partner for developing a quadroma (hybridoma x hybridoma). YP4, a cell line secreting anti-HRPO MAbs (IgG2a) was a rat hybridoma that was previously selected for resistance to 8-azaguanine (Suresh et al., 1986 a & b). Consequently it is sensitive to aminopterin. The YP4 cell line was used as a fusion partner for developing a trioma (hybridoma x splenocyte).

3.2.3. Development of Anti-phage/Anti-AP Quadroma

Two hybridoma cells, in logarithmic growth phase, were maintained in standard RPMI medium containing 10% FBS. The viability for these two cell lines were observed to be over 90% by trypan blue staining. The cells were washed separately 3 times with FBS free RPMI medium. The 2 x 10^7 cells (both P93.11 and P93.8) were resuspended individually in 2 mL of FITC (0.5 μg/mL in serum free medium pH 6.8). The same amount of P92 cells were resuspended in 2 mL of TRITC (1.5 μl/ml in serum free RPMI medium pH 7.4). These cells were incubated in a 37°C water bath for 30 min covered with aluminum foil. After washing once with serum free medium and twice with the electrofusion buffer (0.3 M glucose, 0.1 mM Ca^{2+} and 0.1 mM Mg^{2+}), the cells were resuspended in the same electro-fusion buffer at a density of 1 x 10^7 cells/mL. Fusions for P93.11 x P92 and P93.8 x P92 were performed separately. A 200 μl aliquot of the well mixed cells (2 x 10^6 cells from each labeling group) was added to an electrofusion cuvette. After the cuvette was cooled in ice for at least 5 min, the "safety stand" was connected with the electro cell manipulator, and the cuvette containing cells was placed in the "safety stand". The electro-fusion was performed under the following conditions: (alignment setting) 30 sec, 200 V/cm, 60V of AC, and (fusion setting) 3 pulses of 15 microseconds with field strength 1000 V/cm and amplitude 200V. After the fusion, 1 mL FBS was added to the cells that were allowed to stand at RT for 5 min. Then, cells were transferred to a small flask containing standard RPMI medium supplemented with 20% FBS. These cells were incubated at 37°C, 5% CO_2 for 6 hr before FACS sorting.

The fused cells with double fluorescence signals were selected by FACS and seeded into 96 well culture plates at 1 cell/ per well. These cells were cultured in the RPMI 1640 media containing 20% FBS and 10% of growth factor in humidified 37°C incubator supplemented with 5% CO_2.

3.2.4. Screening for Quadromas Secreting BsMAbs

A one-step bridge ELISA (Suresh, 1986a) was employed for screening the quadroma one week after the fusion. Briefly, this ELISA method only detects bsMAbs by bridging virus coated solid phase antigen with solution phase enzyme (Fig.2.1B). The microtiter plate was coated with 10^{10} pfu $CsCl_2$ purified phages in PBS overnight at 4°C and the plate was blocked by 5% skim milk at RT for 2 hr. After washing, 100 µl of bsMAb containing culture supernatant from different clones (1:4 dilution in Tricine buffer containing 1% skim milk) plus 2 µg AP was added into each well and incubated for 1 hr. After washing, the reaction was revealed by adding 100 µl of pNpp at 1.5 mg/mL in glycine buffer (pH 10.4). The plate was incubated for 20 min and optical density (O.D.) was measured at 405 nm. Positive quadroma cells were transferred to 24-wells culture plates followed by 6-well culture plates. After being retested for bsMAb positivity, these clones were frozen in liquid nitrogen. Quadroma P106.5B and P106.2D were selected for cloning and recloning by limiting dilution based on their initial high signal in the bridge ELISA.

3.2.5. Development of Anti-phage/Anti-HRPO Trioma

A balb/c mouse was immunized with the $CsCl_2$ purified phage M13 following the same procedures described in Table 2.1. HAT sensitive YP4 hybridoma cells were fused with splenocytes from immunized mice using PEG according to same procedures described in Section 2.2.2.3. After fusion, cells were seeded in 7 plates at a concentration of about 2×10^5 cells per well.

3.2.6. Screening for Triomas Secreting BsMAbs

A one-step bridge ELISA (Fig. 2.1B) was employed for screening triomas after two weeks of fusion. The principle is the same as described in Section 3.2.4. In brief, 100

μl of culture supernatant (1:4 dilution in PBS containing 1% skim milk) plus 10 μg/mL of HRPO was added and incubated for 1 hr. After washing, signals were developed following the procedures described in Section 2.2.2.2. The positive trioma clones were transferred to 24-well culture plates followed by 6-well culture plates. After being retested for bsMAb positivity, these clones were frozen at either -80°C or in liquid nitrogen. Three clones P107.7C4, P107.8D8 and P107.11F4 were chosen for cloning and recloning based on their consistently high ELISA activity in cell culture.

3.2.7. Purification of Anti-phage M13/Anti-AP BsMAbs

The P106.5B bsMAbs were purified from anti-phage M13 monospecific MAb (P93.11) contaminants in mouse ascites by the Mimetic blue AP A6XL adsorbent based on the high affinity of Mimetic blue AP ligand for calf intestinal AP and its subsequent mild elution by phosphate (Linder, 1989). It was possible, then, to purify P106.5B complexed with AP and these bound P106.5B-AP complexes could also be eluted by phosphate buffer under mild conditions (Fig.3.1). Briefly, bsMAb containing mouse ascites and crude AP were dialyzed against 10 mM Tricine buffer (pH 8.6) to remove phosphate completely. About 50 mg crude AP in 10 mL of 10 mM Tricine buffer was incubated with 5 mL Mimetic Blue A6XL affinity ligand pre-equilibrated with 10 mM Tricine buffer at 4^0C for 2 hr. The ligand was transferred to a column and any unbound AP and contaminants were washed out by 10 mM Tricine buffer in the column. Subsequently, the Mimetic blue ligand with the bound AP was taken from the column and incubated with 10 mL diluted mouse ascites (1:10) in 10 mM Tricine buffer at 4^0C for 2 hr. The unbound proteins were washed away by 10 mM Tricine buffer in the same column. Finally, the bound Ab/AP immune complexes were eluted by a wash of 10 mM Tricine buffer containing 10 mM potassium phosphate. The eluted Ab/AP complexes included P92 MAb-AP and P106.5B bsMAb-AP immune complexes. The anti-M13 (P93) monospecific MAbs were washed out as an unbound fraction. The eluted fractions were analyzed by both ELISA and SDS-PAGE for bsMAb activity and antibody purity.

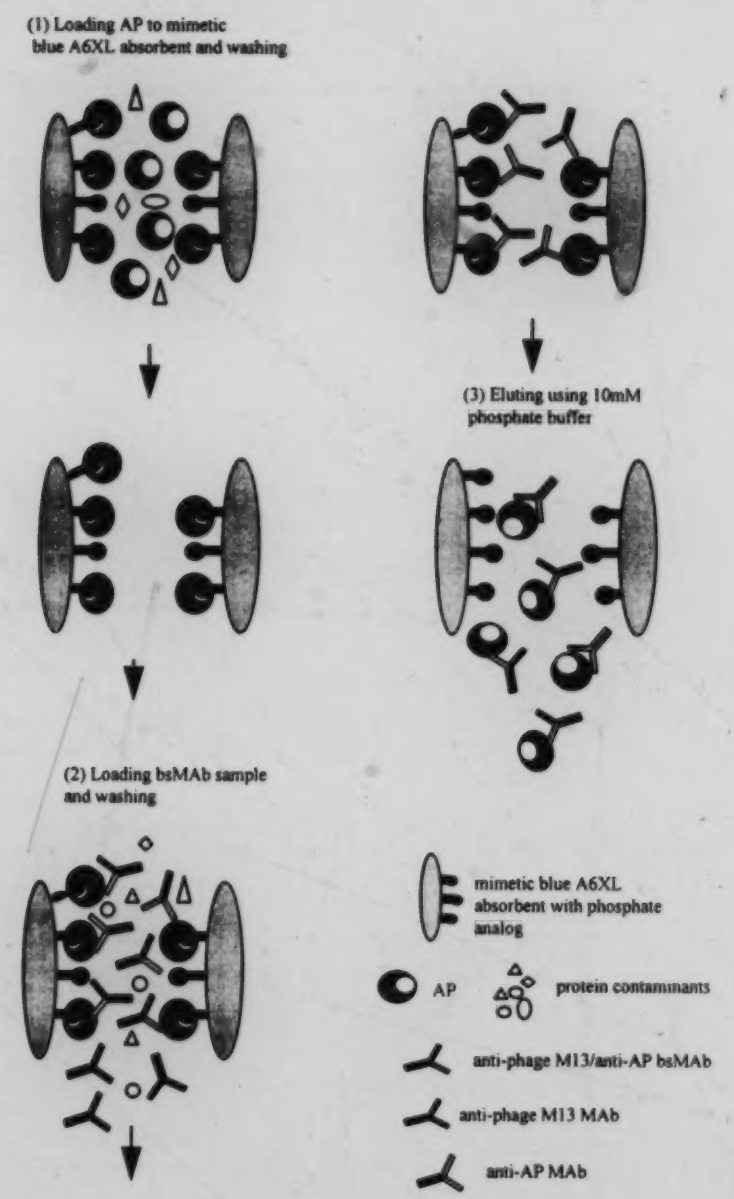

(1) Loading AP to mimetic blue A6XL absorbent and washing
(3) Eluting using 10mM phosphate buffer
(2) Loading bsMAb sample and washing
mimetic blue A6XL absorbent with phosphate analog
AP
protein contaminants
anti-phage M13/anti-AP bsMAb
anti-phage M13 MAb
anti-AP MAb

Fig.3.1 Schematic representation of purification of P106.5B bsAMb-AP immune complexes using mimetic ligand A6XL absorbents

3.2.8. Optimization of ELISA

Since the three anti-phage M13 (Polyclonal Ab HRPO conjugates, P93.11 MAb and P106.5B bsMAb-AP complexes) were prepared in different concentrations, it was necessary to optimize the amount of the three antibodies for their uses in an ELISA. After normalization, these three antibodies could be compared further sensitivities for detecting phage.

Optimal conditions were determined using a streptavidin coated capture ELISA (Fig.2.1C). An ELISA plate was coated with 100 µl of streptavidin at 10 µg/mL overnight at 4^0C and the plate was blocked with 3% dialyzed BSA (to remove any free biotin) for 2 hr. After washing with PBST, 100 µl of crude *E.coli* culture supernatant containing fd phage displaying a biotin-mimetic peptide was added to the individual wells and incubated for 2 hr followed by a wash step. To determine the optimal dilution for each antibody, 100 µl of one of the three antibodies at different (log) dilutions was added for a 1 hr incubation followed by washing. For MAb assay wells, goat anti-mouse HRPO conjugates were added and incubated for an additional 1 hr. The amount of substrate was kept constant in all dilutions wherein ABTS at 0.3 mg/mL was added to goat anti-M13 polyclonal Ab and P93 MAb assay wells and pNpp at 1.5 mg/mL was added to P106.5B bsMAb assay wells to develop the signal. Antibody incubation time and signal development time were kept constant in the assays.

3.2.9. Comparison of BsMAbs with MAbs and Polyclonal Abs in Detecting Phage

To further test the efficiency of bsMAb purification by Mimetic blue AP A6XL adsorbent and the ability of bsMAbs in detecting phage, streptavidin coated capture ELISA was employed for comparing the sensitivity of the three anti-phage M13 antibodies in detecting phage from crude *E.coli* culture supernatant. Each assay was performed under the optimal conditions obtained from the experiments described in section 3.2.8.

The ELISA was performed following the same procedures described in section 3.2.8. Three antibodies were used at an optimal dilution wherein polyclonal Ab HRPO conjugates were at a 1:2500 dilution, P93.11 MAb at a 1:1000 dilution and P106.5BbsMAb at a 1:50. dilution. Signals and backgrounds from these three antibody assays were compared. Uninfected *E.coli* supernatant was used as the negative control.

3.3. Results and Discussions

3.3.1. Development of Anti-phage M13/Anti-AP Quadromas

In order to develop an anti-phage M13/anti-AP quadroma, both anti-phage M13 and anti-AP primary hybridomas were required. P92 and P93.11 were two pre-established primary hybridoma cell lines secreting MAbs against AP and phage M13 antigens respectively (Xu and Suresh, 1998 and Chapter 2).

Electro-fusion was used to fuse the two primary hybridomas instead of traditional PEG-fusion. Electro-fusion is a noninvasive, nonchemical method which has a 50-100 fold higher fusion rate than that of PEG-fusion (Chang, 1992). It was observed that the hybrids from electrofusion recovered faster than those from PEG fusion in our experiments. In electrofusion, the quadromas required only 7-10 days to grow before screening, compared with the cells growing after PEG fusion which took at least two weeks or more.

Previous to selection of bsMAb secreting quadromas, a required component of cell fusion was the need to introduce a drug selection marker into the individual hybridomas. Introducing an additional drug selection marker in the fusion partners is a labor-intensive procedure that usually takes several months. Markers were a necessity since the two parental hybridomas were all resistant to hypoxanthine, aminopterine and thymidine (HAT)-medium. To select quadromas after fusion, one of the parental hybridomas had to be back-selected for HAT sensitivity. The HAT-sensitive hybridoma was then further modified by inducing drug resistance to ouabain (Baker et al., 1974) or neomycin (Delau et al., 1989). When a HAT resistant-ouabain-sensitive hybridoma was fused with a HAT sensitive-oubain-resistant hybridoma, the fused cells (quadromas) were resistant to both HAT and ouabain. As an alternative to this time consuming procedure, eletrofusion and FACS selection were employed (Kreutz and Suresh, 1998). The basis of this method is the dual fluorescent staining of two parental cells and subsequent selection of double stained "fused" cells by FACS (Fig.3.2). It is also likely that some nonspecific association of two cells could be sorted by the FACS but these would not secrete bsMAbs.

By combining electro-fusion with FACS selection, five quadromas secreting high amounts of bsMAbs were obtained in only 7-10 days. The primary data for selecting bsMAb secreting quadromas are listed in Table 3.1. Since hybrid-hybridomas (quadromas) are highly polyploid (Milstein and Cuello, 1984), these cells could lose chromosomes and eventually stop secreting bsMAbs. To eliminate those quadromas that had lost bsMAb production and could overgrow in the cell culture, the quadromas were rapidly cloned and recloned to select the best hybrid-hybridoma. Recloning was repeated until the quadromas stably produced bsMAb. P106.5B was selected as best clone since it stably presented a high ELISA signal during one and half months of continuous culture. This clone was used for the production of anti-phage/anti-AP bsMAbs.

(A) (B)

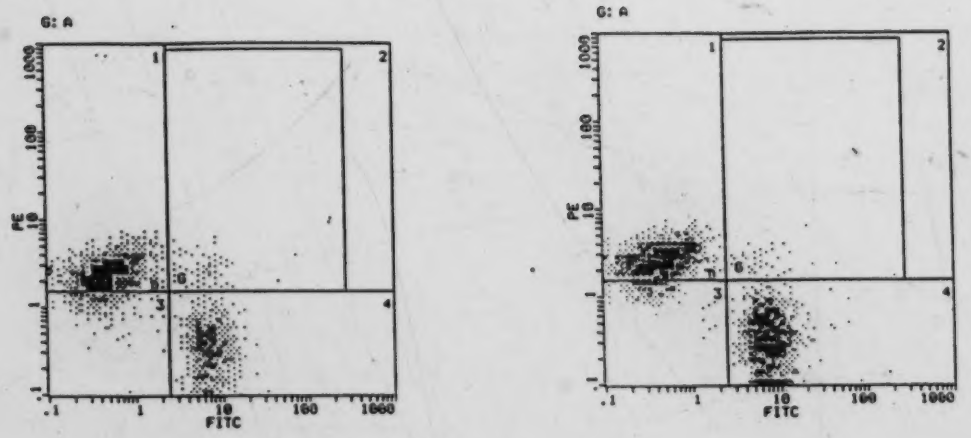


Fig. 3.2 (A) FACS analysis of unfused cells (P93.11 plus P92). Dot plot FACS analysis of a mixture of unfused cells, each dot represents 3 cells. Quadrant 1 contained TRITC labeled cells, quadrant 4 contained FITC labeled cells, quadrant 3 contained the unlabeled cells, and quadrant 2 contained the double positive cells (1.5%). (B) FACS analysis after electrofusion of cells (P93.11 x P92).There was an increase in the number of double positive cells in the quadrant 2 (3.8%). Cells from the area designated as G were chosen to be sorted.

Quadroma clones	Fusion	Screen 1 (O. D.)	Screen 2 (O. D.)	Screen 3 (O. D.)
2D8	p93.8 X p92	0.320	2.895	2.954
5B11	p93.11X p92	1.083	2.568 (P93.11)	2.894
3F5	p93.11X p92	3.481	2.567	1.854
4H5	p93.11X p92	3.173	2.521	2.284

Table 3.1 ELISA for screening anti-M13 phage/anti-AP quadromas. The cell was growing in 96-well culture plate. Screen 1 started 7 days after the fusion. A 100 µl aliquot of culture supernatant from different clones (in a 1:4 dilution) plus AP (20 µg/mL) were added to a $CsCl_2$ M13 phage coated ELISA plate. Signals were developed by adding 100 µl pNpp at 1.5 mg/mL. The positive clones were retested after transfered to 24-well culture plate (screen 2) and 6-well plate (screen 3).

3.3.2. Development of Anti-phage M13/Anti-HRPO Trioma

Since HRPO is the most commonly used alternative marker enzyme to AP, anti-phage M13/anti-HRPO bsMAbs would be very useful in the ELISA applications of phage M13. A slightly different approach was used for producing anti-phage M13/anti-HRPO bsMAbs by developing a trioma. A trioma is the cell produced by fusing a hybridoma having specificity for one antigen with a spleen cell from an animal immunized with a second antigen (Suresh et al., 1986b; Cao and Suresh, 1998). For selecting triomas after fusion, the hybridoma to be used for fusion has to be back-selected for HAT sensitivity. This can be achieved by incubating the cells with 8-azaguanine (Kontsekova et al., 1991), which induces HGPRT deficiency by point mutation, then the hybridomas become sensitive to aminopterin. Thus, only hybrid cells (hybridomas x spleen cells) can survive in HAT medium since either fused or unfused spleen cells only grow for a limiting time.

YP4 was a well established rat hybridoma cell line secreting anti-HRPO MAbs and this hybridoma was back-selected for HAT sensitivity previously in our Lab (Kreutz and Suresh 1995). Since electro-fusion is more suitable for fusing a small number of cells, PEG was used for fusing the relatively larger amount of YP4 cells with the spleen cells from $CsCl_2$ purified M13 phage immunized mouse. A one step bridge ELISA was used for screening bsMAb secreting triomas. In total, 28 clones were selected. The primary data for the selection of triomas is listed in Table 3.2.

The bsMAbs produced by the trioma method were slightly different than bsMAbs produced by the quadroma method with one arm of these bsMAbs being from a heterogenous population of spleen cells which secreted uncharacterized antibodies. In contrast, anti-phage/anti-AP bsMAbs were from two well characterized hybridomas which secreted antibodies with known antigenic specificity and affinity.

3.3.3. Purification of P106.5B BsMAbs

The anti-phage M13/anti-AP quadroma secreted not only anti-phage M13/anti-AP bsMAbs (P106.5B), but also anti-phage M13 and anti-AP monospecific MAbs (P93.11 or

Clones	Screen 1 (O. D.)	Screen 2 (O. D.)	Screen 3 (O. D.)	Clones	Screen 1 (O. D.)	Screen 2 (O. D.)	Screen 3 (O. D.)
2G6	0.654	0.451	0.259	8B8	1.261	1.052	0.864
2B10	0.382	0.185	0.054	8D8	1.864	1.458	1.257
2B15	0.841	0.745	0.532	8C11	0.727	0.231	0.095
2D8	0.803	0.813	0.789	8F12	0.635	0.547	0.465
7D10	0.467	0.231	0.108	8B3	1.082	1.036	0.984
7B5	0.988	0.854	0.543	8D5	1.126	0.864	0.465
7C5	1.054	0.985	0.854	11D4	0.437	0.210	0.023
7C4	2.246	2.039	1.986	11D5	1.569	1.358	1.245
7E5	0.757	0.658	0.587	11F4	2.540	2.246	2.159
7D2	1.252	1.024	0.896	11H4	0.587	0.421	0.325
7B8	0.648	0.541	0.235	11F5	0.963	0.847	0.654
8F5	0.886	0.542	0.321	11F2	0.492	0.324	0.258

Table 3.2 ELISA for screening anti-M13 phage/anti-HRPO triomas (P107). Screen 1 started two weeks after the fusion. A 100 µl aliquot of culture supernatants from different clones (in 1:4 dilution) plus HRPO at 10 µg/mL were added to $CsCl_2$ M13 phage coated ELISA plate to assay for the presence of anti-phage M13/anti-HRPO bsMAbs. Signal was developed by adding 100 µl ABTS at 0.3 mg/mL. Positive triomas was retested after transfered to 24-well culture plate (screen 2) and 6-well plate (screen 3).

P92). The former may decrease the sensitivity of the bsMAb based immunoassay by competing with P106.5B for binding sites on phage coated on the ELISA plate. This limitation of quadromas can be overcome by sophisticated genetic engineering methods secreting only bsMAbs (Staerz and Bevan, 1996). However, quadroma development is a simpler and relatively faster method in obtaining bsMAbs for diagnostic applications. Novel purification methods are necessary to resolve the various antibody species.

In our approach, a mimetic blue A6XL resin was exploited to purify P106.5B from P93.11 MAbs. Mimetic blue A6XL resin was known to bind AP in Tris buffer due to the presence of a phosphate analogue present in the blue chromophore (Lindner, 1989). The AP enzyme efficiently binds to this column matrix and can be subsequently eluted with phosphate buffer, a very mild eluting condition compared to other common low pH based elution steps. This column was used to test if the P106.5B could bind to AP pre-absorbed on the affinity matrix and then be eluted in same fashion as free AP. We found that if crude P106.5B bsMAb was dialyzed against Tris buffer, it was efficiently retained and could be eluted as a P106.5B bsMAb-AP complex with a mild phosphate buffer. The principle of Mimetic blue affinity purification is schematically represented by Fig.3.1. Although P92 MAb-AP (the monospecific MAb-enzyme complex) would also be copurified with bsMAb-AP, the presence of the P92 is not a problem in the ELISA assay, so long as excess AP is used in the ELISA assay. This complex would be washed away during the ELISA procedure.

The elution profile in Fig.3.3 shows a majority of P106.5B bsMAb-AP immue complexes were recovered in fractions 26-34 (collected in 1 mL/tube). These fractions corresponded to high bsMAb activity by a direct binding ELISA (Fig.2.1B). Fractions with high antibody activity were checked for protein concentration by SDS-PAGE. The results are shown in Fig.3.4 wherein Lane 3, 4, and 5 represent purified bsMAb-AP immune complexes corresponding to fraction 28, 30 and 32 in Fig.3.3. A Majority of contaminants was removed compared with crude ascites in lane 7. The band at about 50 Kd is the antibody heavy chain and two antibody light chain with slightly different molecular weight are observed at about 25 Kd. AP is represented by the multiple

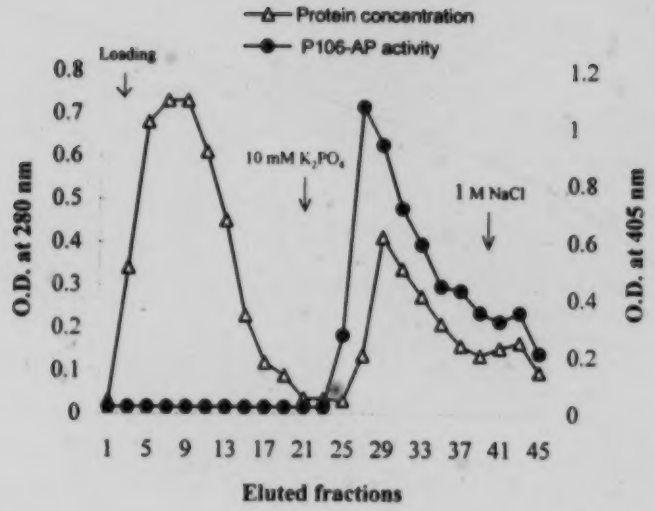


Fig. 3.3 Chromatography of the Mimetic blue A6XL purification of P106.5B bsMAb-AP immune complexes. The samples (AP and mouse ascites containing bsMAb) were incubated in 2 steps with 5 mL Mimetic affinity ligand. Washing procedures were performed between each incubation in the column. The bound fractions included anti-AP P92 MAb-AP and P106.5B bsMAb-AP immune complexes and were eluted with 10 mM phosphate in Tricine buffer (pH 8.5). Fractions of 1 mL were collected and assayed for both protein concentration and P106.5B bsMAb-AP activity.

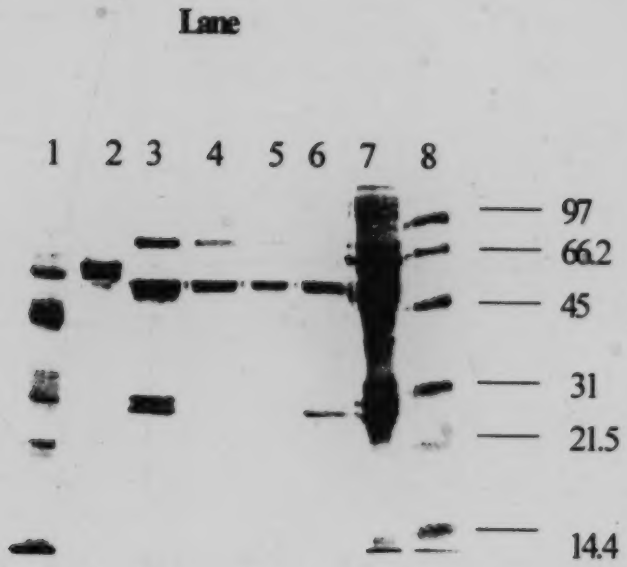


Fig. 3.4. SDS-PAGE analysis of purified P106.5B bsMAb-AP immune complexes: The samples were electrophoresed on 12% SDS poly-acrylaminde gel at 150 V for 1.5 hr and proteins were visualized by Coomassie blue staining. Lane 1: AP from Crude calf intestinal mucosa. Lane 2: commercial pure AP. Lane 3 to lane 5: fractions 28, 30, 32 from Mimetic blue affinity purification containing high bsMAb activity. Lane 6: Purified anti-phage M13 MAb (P93) by DE-52 ion exchange chromatography. Lane 7: Crude ascites containing P106.5B bsMAbs. Lane 8: LMW markers.

bands above the heavy chain band corresponding to 50-70 Kd. This indicates the glycoprotein AP has subunits since the MW of the whole molecule is 100-140 Kd. Fractions 28-32 with both the highest antibody activity and protein concentration were pooled for further studies. The antibody concentration was determined to be 0.28 mg/mL.

Dramatically different P106.5B activities were observed by ELISA before and after purification (Fig.3.5). A bell shaped curve obtained could be explained by the fact that the presence of anti-phage M13 monospecific P93.11 in the crude ascites competing with the bispecific P106.5B and decreasing the signal in the more concentrated ascites sample. The signal increased significantly with the dilution of the cultured quadroma ascites fluid (1:200 to 1:800) due to decreased competition with relatively more M13-binding sites available. However, the signal decreased again with further dilution due to the ultimately low antibody titer. An important observation we made from the development of several other quadromas was that the screening of cell culture supernatant has to be done at two or three different dilutions in order to advoid missing a potential clone. The activity of purified P106.5B bsMAb shows a typical dilution curve. Dilution at 1 to 50 presents the highest signal. This indicate the successful removal of P93.11 and the monospecific MAb which was responsible for the bell shaped curve in ascites fluids.

3.3.4. Sensitivity and Background of Three Anti-phage Abs in Detecting Phage.

To further evaluate the newly developed bsMAb probe, I compared its sensitivity with other two anti-phage Abs (P93.11 MAb and polyclonal Ab HRPO conjugate) in detecting phage from crude *E.coli* supernatants. Prior to this assay, the optimal concentrations of three Abs were determined to obtain a maximum sensitivity for each Ab detection. Three different dilutions for each Ab were tested to determine the optimal dilution. Fig.3.6 shows P106.5B bsMAb-AP at 1:20 and 1:50 giving a similar signal intensity. But the signal was greatly decreased in the wells with phage more than 10^8 pfu when bsMAb-AP immune complexes were further diluted to 1:100. So bsMAb-AP immune complexes were used at a 1:50 dilution. The same principle was used to choose

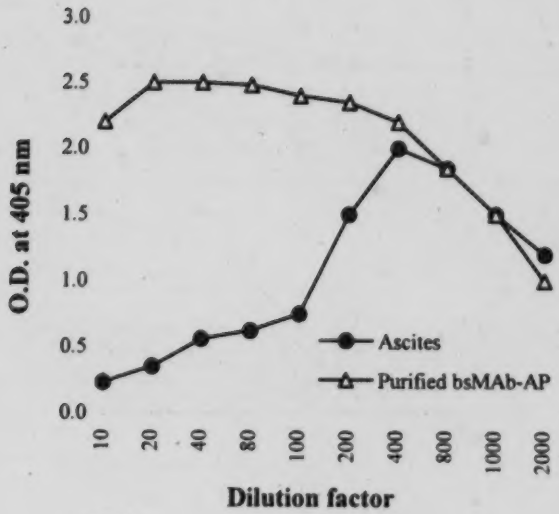


Fig. 3.5 ELISA analysis of the activities of crude and purified P106.5B bsMAb: Direct binding assay on M13-phage coated microtiter plates was performed using crude ascites fluid and the purified bsMAb-AP immune complexes from the Mimetic blue A6XL column. Crude mouse ascites and purified bsMAb-AP immune complexes (0.28 mg/mL) were diluted as shown. A 100 µl from each dilution plus 2 µg AP was added to the ELISA wells coated with $CsCl_2$ gradient purified phage M13 and incubated for 2 hr. The amount of bsMAb was detected using pNpp as a substrate.

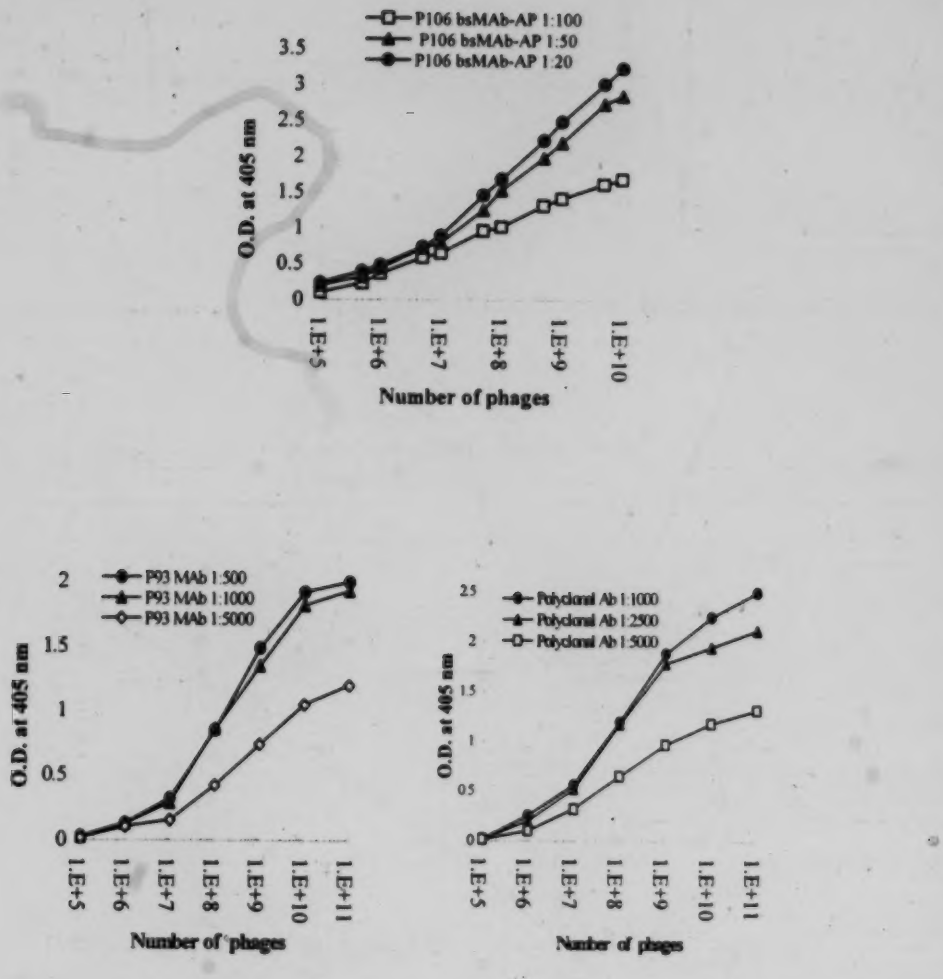


Fig. 3.6 Determination of the optimal dilution of three types of anti-phage antibodies for a streptavidin capture ELISA: Various dilutions of P106.5B bsMAb-AP complexes, P93.11 MAb and Polyclonal Ab HRPO conjugates were tested with different amount of fd phage displaying biotin mimetic peptide, which were captured by streptavidin immobilized on a microtiter plate. For bsMAb and polyclonal Ab assay wells, signals were developed by directly adding substrate pNpp at 1.5 mg/mL and ABTS at 300 μg/mL respectively. For MAb assay wells HRPO conjugated goat anti-mouse second antibodies were added and incubated before the signal was developed with ABTS at 300 μg/mL.

the optimal dilution for the MAb and the polyclonal Ab based on ELISA results wherein MAb could be used at 1:1000 dilution and polyclonal Ab at 1:2500 dilution .

Detection sensitivities of the three anti-phage Abs were compared on streptavidin coated plates while each Ab was used in optimal dilution. Phages displaying a biotin mimetic peptide can be captured by streptavidin immobilized on plate. The captured phage could be detected by the P106.5B bsMAb-AP immune complex, the P93.11 MAb and second Ab HRPO conjugate, or polyclonal Ab HRPO conjugates. The Ab that showed highest signal at the lowest phage concentration would have the highest sensitivity. Fig.3.7 showed that the bsMAb-AP immune complexes were able to detect 10^5 phage and both P93.11 MAb and polyclonal Ab were able to detect 10^6 phage. Polyclonal Ab gave higher signal than MAb in detecting 10^6 phages. The data were plotted after deducting background signals, which were obtained using crude *E.coli* supernatant without phage infection as controls for nonspecific binding. P106.5B bsMAb also had lower background signal compared with both the P93.11 MAb and the polyclonal Ab (Fig.3.7 inset).

In earlier studies (Bhardwaj et al., 1995), a similar affinity capture phage ELISA was developed using anti-phage monospecific MAb to detect phages displaying the B domain of staphylococcal protein A captured in an IgG coated well. We found in our experiments that bsMAb had obvious advantages over MAb and polyclonal Ab when the number of phages was lower and crude phage sample with a high number of contaminating proteins was used. This is because the bsMAb is designed with two predetermined binding sites in a single molecule. This antibody design can uniformly reproduce 1:1 binding with signal generating molecule and present a high specific activity (Kreutz and Suresh 1997). Chemically modified antibody conjugates are generally more sticky, giving higher non-specific binding due to aggregation and the non-uniform size of the antibodies. Recently, immunohistochemical methods have also been used to study the binding of phage displaying desired peptides with their cogent target on whole cells or tissue sections (Nissim, 1994; Poloni, 1997). An anti-phage/anti-AP bsMAb could also be used as a powerful tool in these techniques. In earlier studies, a bsMAb used in

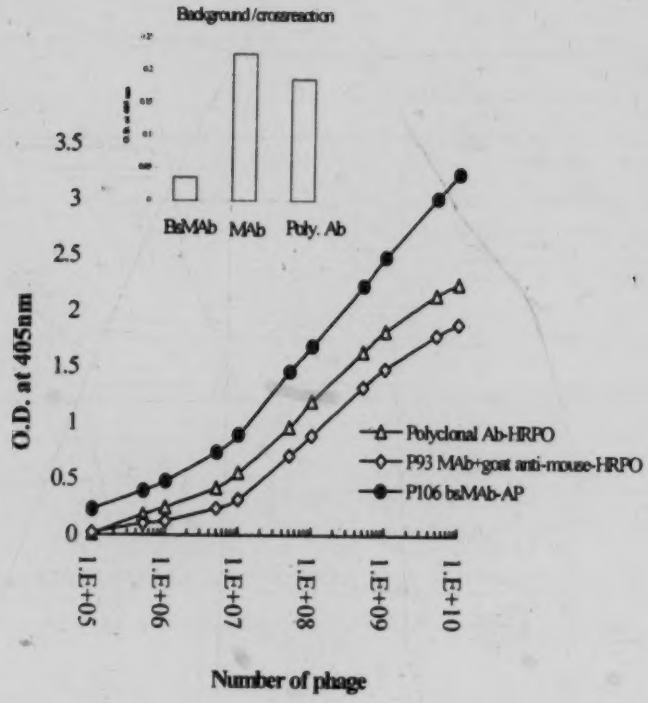


Fig. 3.7. Comparison of three anti-phage antibodies in detecting fd phages displaying biotin mimetic peptide: The crude culture *E.coli* supernatant containing fd phages was added to the streptavidin coated plate at different concentrations. The affinity captured fd phages were detected by P106.5B bsMAb-AP immune complexes or P93.11 MAbs or polyclonal Abs. Each point was performed in duplicate and the value was calculated by subtracting background absorbance. The background of the three anti-phage antibodies was checked using uninfected *E.coli* supernatant.

immunocytochemistry led to major improvments in sensitivity, signal-to-noise ratio and simplification of staining procedures (Milstein and Cuello, 1984).

Chapter 4. Applications of Anti-phage M13/anti-AP BsMAb in ultrasensitive Virus ELISA; Phage Display Technology and Viral Purification

4.1. Introduction

BsMAbs are uniquely engineered antibodies with intrinsic binding to any two predetermined antigens (Suresh et al., 1986a; Cao and Suresh, 1998). These molecules can bind an antigen (e.g. tumor, bacterium or virus) with one paratope and a diagnostic or therapeutic entity with another paratope. This novel immunoprobe assures that every antibody molecule is associated with a signal generating molecule, drug or toxin for a maximal specific activity and functional efficiency (Kreutz and Suresh, 1997). Therefore, problems caused by chemical cross-linking of enzymes or drugs to with monospecific MAbs can be avoided. BsMAbs have been prepared for a number of applications, such as immunoassays (Kreutz and Suresh, 1997 and 1995), immunohistochemistry (Suresh et al., 1986b) and immunotherapy (Wong and Covin, 1987; Staerz and Bevan, 1989).

Detection of viral antigens and the measurment of viral load present in body fluids would be a desirable diagnostic or monitoring tool for many infectious diseases instead of measuring host antibodies to these pathogens. Host antibody measurement is not capable of distinguishing between an active infective state and a previous history of infection (Mok et al., 1987). This is because the titer of host antibodies in serum persists long after the disease state. In the case of HIV infection, an HIV antigen assay could be used for measuring viral load to determine the prognosis of the infected individual and to monitor the effectiveness of anti-viral therapy (Mellors et al., 1996). However, current ELISAs for detecting HIV antigens such as the p24 antigen assay lack the sensitivity to be used in situations as described above (Dwyer et al., 1996). There is a clearly need to develop more inexpensive and sensitive ELISA methods to detect HIV

and other viral or bacterial antigens. These could serve as alternatives to measuring host antibody titers and more complex PCR techniques.

In this chapter, we demonstrate the usefulness of this new bsMAb immune probe in three different areas: (1) development of an ultrasensitive ELISA for rapid virus detection using phage M13 as a model, (2) a bsMAb based ELISA for rapid screening of a phage peptide library and (3) a bsMAb based affinity chromatography with a mild elution for recovery of active viruses.

4.2. Materials and Methods

4.2.1 Materials

NADP (NAD free) was from Boehringer Mannheim (Germany). fluorescein isothiocyanate (FITC); tetramethyl rhodamine isothiocyanate (TRITC); alkaline phosphatase (E.C.3.1.1.1) from calf intestinal mucosa (50U/mg, solid); diaphorase; alcohol dehydrogenase (ADH); p-iodonitrotetrazolium violet (INT-violet); para-nitrophenyl phosphate (pNpp) and diethanolamine were purchased from Sigmal Chemical Co.(St. Louis. MO, USA); *E.coli* K91 and the fd phage peptide library with random decapeptide inserts were kindly provided by Dr. Malcom, Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta. Fd phage displaying a biotin mimetic peptide was provided by Dr. Jacobs, Biomira Inc Edmonton. The phage peptide library used in our experiments was constructed by mixing fd phage displaying a biotin mimetic peptide with fd phage displaying random decapeptides in a 100 pfu to 1.4 $x10^{11}$ pfu ratio (by plaque assay).

4.2.2. Antibodies and hybridomas

P93 is a murine hybridoma secreting anti-phage M13 monospecific MAbs (described in Chapter 2 as P93.11) and purified by DE-52 ion exchange chromatography to a concentration of 1 mg/mL. P106 is a quadroma secreting anti-phage M13/anti-AP

bsMAb (described in Chapter 3 as P106.5B) and purified by Mimetic blue A6XL affinity absorbent as bsMAb-AP immune complexes to a concentration of 0.28 mg/mL. Goat anti-phage M13 polyclonal antibody HPRO-conjugate was from Pharmacia, Canada.

4.2.3. Development of an ultrasensitive ELISA for detecting viral antigen

4.2.3.1. Enzyme amplified ELISA

A microtitre plate was coated with DE-52 purified P93 (2μg/well) overnight at 4°C. The plate was blocked with 5% skim milk in PBS (pH7.4). A 100-μl aliquot of the phage with a varying concentration ranging from 10^2 pfu to 10^6 pfu was added to each well in triplicate and the plate was incubated at RT for 3 hr. The plate was then washed three times with PBS-T (PBS containing 0.05% Tween-20). A 100-μl volume of purified P106-AP immune complexes (2.8 μg/well) was added and incubated for another 1 hr at RT followed by a extensive washing step. A 50 μl aliquot of 0.2 M NADP in 0.9 M diethanolamine (DAE), pH 9.5, containing 1 mM $MgCl_2$ was added and incubated for 15 min at RT. The amplification cycle was started by adding 110 μl of a cycling solution containing: 50U/mL ADH, 2.5U/mL diaphorase and 0.55 mM INT in 25 mM sodium phosphate buffer, pH7.2 including 4%(v/v) ethanol (Cook and Self, 1993). The enzyme reaction was stoped after a 20 min incubation by adding 50 μl of 0.4 M HCl. The optical density was recorded at 492 nm using a microtiter plate reader.

4.2.3.2. Conventional ELISA

In the same ELISA plate, a conventional ELISA was performed in triplicate for comparison. After a final washing step, a 100 μl aliquot of 1.5 μg/ml pNpp in 0.9 M diethanolamine buffer, pH 9.5, containing 1 mM $MgCl_2$ was added to each well and incubated for 60 min at RT. The optical density at 405 nm was recorded.

4.2.4. Screening phage peptide library for phage displaying biotin mimetic peptide

4.2.4.1. Detection of target binding phage by streptavidin capture ELISA

This assay format is schematically presented in Fig.2.1C. An ELISA plate was coated with 100 µl of streptavidin at 20 µg/mL overnight at 4^0C and the plates were blocked with 3% dialyzed BSA (to remove any free biotin) in PBS for 2 hr. After washing with PBS-T, a 100 µl aliquot of crude cultured *E.coli* supernatant containing amplified eluates from the panning of phage peptide libraries in a serial log dilution was added to individual wells and incubated for 2 hr followed by another wash step. A 100 µl aliquot of one of the three anti-phage antibodies was added, namely, goat polyclonal HRPO conjugate (1:2500), P93 MAb anti-M13 (1:1000) or P106 bsMAb-AP enzyme complex (1:50) plus an additional 10 µg/ml of AP. After washing, HRPO conjugated goat anti-mouse second Abs were added to assay wells wherein P93 monospecific antibody was used and incubated for additional 1 hr. For signal development, 100µl of ABTS (300 µg/mL) was added to both the P93 and polyclonal Ab assay wells and 100µl of pNpp (1.5 mg/mL) was added to the P106 assay wells. In a control well, *E.coli* supernatant containing wild type phage was used.

To determine the crossreaction (background) of the three anti-phage Abs with *E.coli* proteins, all procedures for ELISA were repeated as above with the exception of uninfected *E.coli* supernatant being added to a streptavidin coated plate instead of *E.coli* supernatant containing phage.

4.2.4.2 Phage standard curve by sandwich ELISA

To develop a phage standard curve, crude cultured phage solution (10^{12} pfu/mL). was diluted in a serial log fashion from 1:10 to $1:10^{12}$. A 100µl volume from each dilution was used for the phage sandwich ELISA using an anti-phage M13 P93 MAb coated ELISA plate (Fig.2.1D). The bound phages were detected following the same

procedures described in Chapter 3.2.9 for the P106.5B bsMAb assay. The standard curve was plotted as pfu's *vs* ELISA signal.

4.2.4.3. *Biopanning of phage peptide library*

In the initial panning, 6-well culture plates were coated with streptavidin at 100 μg/ml and blocked with 3% dialyzed BSA in PBS. The 1.4×10^{11} fd phages from the spiked phage peptide library in 1 mL PBS-T was added to the plates. After 30 min of incubation, the wells were washed with PBS-T. A two step method was used for eluting bound phage: the plate was incubated with 1 mL of 0.5 mM biotin for 5 min, washed with PBS-T, then incubated with another 1 mL of biotin for 30 min. The phages in the second elution were amplified in *E.coli* K91 in the presence of 10 μg/mL tetracycline. Amplified phages were concentrated by PEG/NaCl precipitation. The titer of phage was determined by a M13 sandwich ELISA as described in section 4.2.4.2 and compared with the standard curve and conventional plaque counting. This value was used to calculate an input volume corresponding to 2×10^{11} pfu for the next round of panning. This panning-amplification process was repeated four times over the course of 5-6 days. The crude phage culture supernatant from each amplification was analyzed by a streptavidin capture ELISA for monitoring enrichment of biotin mimetic phage. The signal from the streptavidin coated wells minus the signal from control wells indicated the specific binding of the biotin mimetic phage and the increase in signal with each successive cycle of amplification represented data showing the enrichment of the biotin mimetic phage. After the fourth panning and elution, phages were plated at low density in LB plates. Fifty individual plaques were randomly selected for ELISA analysis and 5 clones were chosen for DNA sequencing to confirm the biotin mimic peptide sequence similarity with the original biotin phage spiked in the phage display library (Devlin et al., 1990).

4.2.5. BsMAb based affinity method for purification of viruses

4.2.5.1. Microtiter plate method for viral purification

A 96 well microtitre plate was coated with 200 µl of AP at concentration of 20µg/mL overnight at 4°C. After blocking with 3%BSA for 2 hr, 200 µl of mouse ascites containing P106 bsMAb at a 1:200 dilution was added into the microtiter plate and incubated for 2 hr. Then, free Abs were washed out and 200 µl of PEG purified phage suspension from 10^6 pfu/mL to 10^{10} pfu/mL were added and incubated for another 2 hr. The unbound phages were washed out and bound phages were eluted by adding 200 µl of AP at 100 µg/mL followed by incubation for 2 hr. The eluted phages were quantified by a plaque assay. The control plate was performed using the same procedure after directly blocking with 3%BSA and no AP coating.

4.2.5.2. Mimetic Blue absorbent column method for viral purification

Fig.4.1 schematically describes the Mimetic Blue absorbent column method for viral purification. All samples (calf intestinal AP, mouse ascites and PEG purified phage) were dialyzed for 12 hr at 4°C against 2 L of 10 mM Tricine buffer with three changes. Five milliliters of AP (5 mg) was incubated with 1.5 mL of mimetic blue AP A6XL absorbent pre-equilibrated with Tricine buffer 2 hr at RT with gentle shaking. Then the absorbent was transferred to a 5 mL column. The column was washed with same buffer until the protein concentration reached the base line at 280 nm. The absorbent was taken out and incubated with a 5 mL bsMAb sample for 2 hr. The absorbent was transferred to the column followed by washing under UV monitoring at 280 nm. Finally, the absorbent was incubated with 3 mL of PEG purified phage sample. for 2 hr. After extensively washing in the column, AP-bsMAb-phage complexes were eluted with 10 mL of 10 mM phosphate in Tricine buffer (pH 8.5). Fractions were collected for a plaque assay. The control column for the purification of M13 was done using the same procedure without adding bsMAbs to the Mimetic Blue column.

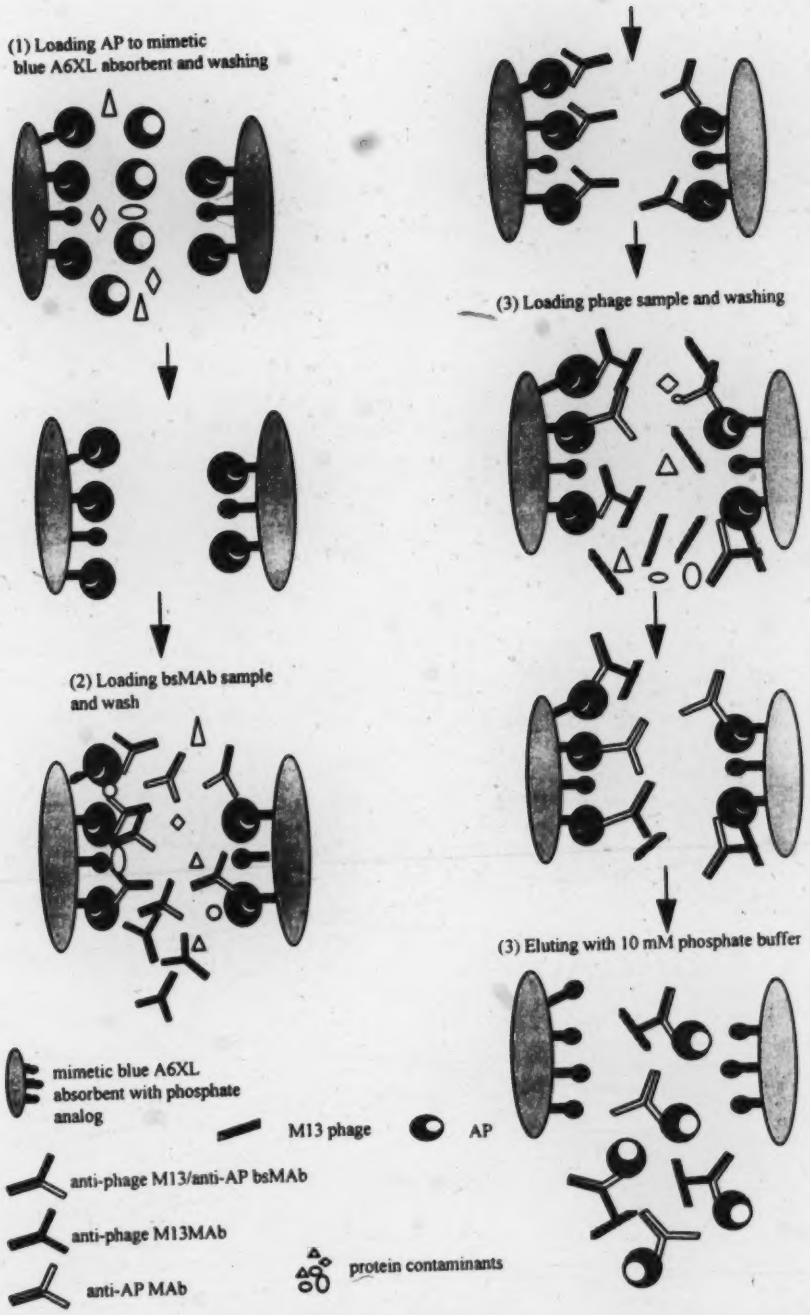

(1) Loading AP to mimetic blue A6XL absorbent and washing
(2) Loading bsMAb sample and wash
(3) Loading phage sample and washing
(3) Eluting with 10 mM phosphate buffer
mimetic blue A6XL absorbent with phosphate analog
M13 phage
AP
anti-phage M13/anti-AP bsMAb
anti-phage M13MAb
anti-AP MAb
protein contaminants

Fig.4.1. Schematic representation of purification of viruses using mimetic blue A6XL absorbents

4.3. Results and Discussions

4.3.1. Enzyme amplified sandwich ELISA for detecting viruses

The bsMAb sandwich ELISA for M13 was developed using purified P93 anti-M13 monospecific MAb as the solid phase capture reagent. Various amount of $CsCl_2$ purified M13 phage was incubated and subsequently the bsMAb-AP immune complexes were added to detect the viruses. Signal generation was either by conventional pNpp or by the cyclic enzyme amplification method. The enzyme amplification method was compared with the conventional ELISA method in detecting the viruses as described in section 4.2.3. Optimal conditions for both methods were obtained by varying concentration of antibodies and incubation time (data not shown). The practical sensitivity of the amplified assay was found to be 100 phage particles (1000 M13/mL) which is equal to 2.3 fg (23 fg/mL) phage major coat proteins. In the conventional assay it was 10^5 phage (10^6 M13/mL) which was approximately equal to 2.3 pg (23 pg/mL) of phage major coat proteins. Thus, a 1000 fold increase in sensitivity was achieved by the enzyme amplified ELISA when compared with the conventional ELISA (Fig.4.2). Analytical Sensitivity (Theoretical detection limit) was less than 100 phage particles which was determined by the mean of 20 replicates of the zero standard plus 2 SD (Table 4.1). However, the signal from 10 to 100 phage particle increased in a nonlinear fasion. This could be due to a variety of factors including enzyme avtivity and antibody affinity in very low concentration sample as well as the influence of low concentration of phages being saturated with relatively high level of adsorbed solid phase antibody putatively generating some competition with bsMAb. Functional Sensitivity (Practical sensitivity) was 100-500 phage particles which was determined from the precision (<20%) of very low concentration of virus samples (Table 4.1). In an effort to develop a highly sensitive ELISA as a routine viral screening assay or viral load assay, bacterial phage M13 was chosen as a model virus to demonstrate feasibility since they were relatively safe and were conveniently available. In this assay, viruses were captured by P93 monospecific MAbs on the ELISA plate, then detected by P106-AP immune complexes coupled with

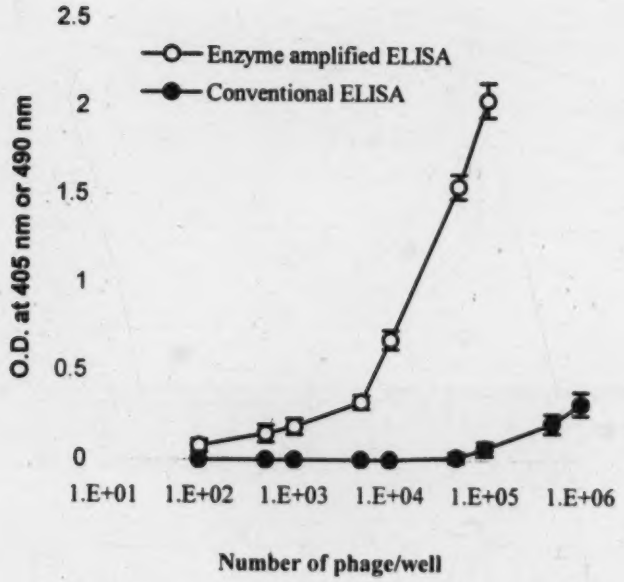


Fig. 4.2 Comparison of the conventional and enzyme amplification ELISA for sensitivity of detecting phage M13: ELISA plate was coated with DE-52 purified anti-phage P93 MAb. A 100µl aliquot of phage M13 from log serial dilutions was added and incubated for 3 hr. Purified P106 bsMAb-AP immune complexes together with additional AP was added and incubated for 1 hr to detect captured phage particles. Signals were developed by adding either pNpp as a conventional substrate or by an enzyme amplified method (section 4.2.3.1). Optical density at 405 nm or 490 nm was plotted vs phage concentration. Each point represents the mean of three determinations.

Number of phages in the ELISA well	Mean (O. D.)	S. D (% of mean)
zero (n=20)	0.775	0.028 (3.6%)*
10 (n=5)	0.840	0.025 (2.1%)
50 (n=5)	0.847	0.046 (5.4%)
100 (n=5)	0.852	0.030 (3.5%)
500 (n=5)	0.887	0.065 (7.3 %)

* mean + 2 S.D =0.831

Table 4.1 Sensitivity or lowest detection limit of enzyme amplified ELISA. Analytical sensitivity (Theoretical detection limit) was determined by the mean of 20 replicates of the zero standard plus 2 SD. The mean value from 10 to 100 phage assay well showed above 0.831 (20 replicates of the zero standard plus 2 SD). Functional Sensitivity (Practical sensitivity) was determined from the precision (<20%) of very low concentration samples. Low concentration phage samples (10, 50, 100 and 500 phage particles) showed the SD range from 2.1% to 7.3% of mean value.

an enzyme amplified method (Cook and Self, 1993). This unique design has two advantages over the current conventional ELISA. Firstly, bsMAbs instead of enzyme-conjugated MAbs were used in the second step of the assay. This new immunoprobe P106 was able to bind an AP molecule with one paratope while its other paratope binds an epitope on the M13 phage coat protein. Given that coat proteins in all viruses are present in multiple copies (often several hundred), it is likely that multiple capture and tracer antibodies are involved in the sequestering of the virus. Increased avidity and amplified detection are therefore possible. This uniform 1:1 ratio of bsMAb to enzyme ensures the intensity of the signal is at the theoretical maximum for such ELISA's. In the case of the monospecific MAb conjugates, a small fraction of either Ab or enzyme could be inactived due to chemical manipulation and aggregate formation. This may partly explain why the enzyme amplified ELISA described previously (using enzyme-conjugated MAbs) only obtained a 40 fold increase in sensitivity compared to a conventional ELISA (Cook and Self, 1993). Secondly, signal generating AP was not used to produce color products directly, but used as a catalytic activator to start an amplification cycle of color production. In this system, AP converts NADP into catalytic activator NAD which initiates the NAD specific redox cycle wherein NAD is reduced by NAD specific ADH and subsequently, the oxidized form is regenerated by diaphorase with the concomitant reduction of INT-violet to an intensely purple formazan as the end product (Fig.1.5). It is estimated that each primary product NAD molecule could generate 600 secondary colored products by entering the redox cycle. Thus the estimation of viral load could be determined by measuring the absorbance of the secondary color products.

The sensitivity of the enzyme amplified ELISA could potentially be improved if polyalkaline phosphatase was used instead of the native enzyme (a polyperoxidase homopolymers with 10-80 monomers cross-linked into one large molecule was recently introduced by Research Diagnostics, Flanders, NJ). Alternatively the sensitivity could be pushed to detect individual virus particles by laser induce fluorescence detection of virus bound AP adapting techniques previously described to detect a single molecule of AP (Craig et al., 1996). Combining bsMAbs and such sophisticated detection methods could provide alternative technology to PCR strategies in the applications of viral load

detection/estimation of AIDS, hepatitis and other viruses. This has become important because in a recent study, plasma viral HIV load was found to be a better indicator of prognosis than the $CD4^+$ lymphocyte count (Mellors et al., 1996). Viral load not only accurately correlates to disease progression but also offer clinicians a valuable tool in evaluating a patient's response to antiviral therapy. However, the sensitivity of current HIV antigen ELISAs is too low to be used for this purpose (Dwyer et al., 1996). The current commercially available viral load assay measures the concentration of HIV-1 RNA and has a sensitivity of 500 copies/ml (Dwyer, et al., 1996). It is also time consuming and costly method compared with the well accepted ELISA technology. Refinement of the bsMAb ELISA's could provide a low-cost sensitive alternative to measureing viral loads of several medically important viruses. More recently, a novel amplified ELISA using tyramide-biotin reporter deposition (Boni et al., 1997) has been adopted to allow detection of HIV antigens at very low levels (200 fg/ml).

4.3.4. Utilities of bsMAb in improving biopanning peptide from phage peptide library

Phage display technology is an efficient and prolific means to generate large numbers of diverse peptides and proteins and select the molecules that perform useful functions (Cannon et al., 1996). Filamentous phages (fd, f1 and M13) have found widespread use as cloning vectors for phage display technology. The genetic information encoding novel proteins and peptides are inserted into the genome of M13 bacteriophage, and the expressed products are displayed on the surface of M13 fused to one of the phage coat or tail proteins. In general, these vectors have been used to display large numbers of random peptide sequences for selecting or panning enzyme inhibitors (Robert et al., 1992), receptor agonists and antagonists (Doorbar and Winter., 1994), enzyme variants or antibody variants, improved (or novel) catalytic activity or improved binding properties (Marks et al., 1992; Lowman and Well, 1993).

Biopanning (Scott and Smith, 1990) has been widely used for screening phage-display libraries in order to select a desired peptide or protein variant capable of binding to a given antigen. This method requires several phage purification cycles and plaque

assays that are time consuming and labor intensive. A lot of effort has been made by researchers to simplify this procedure with immunological methods (Christian et al., 1992; Valadon and Scharff, 1996). Comparing these methods, the reverse ELISA was more sensitive in detecting phage displaying peptide with lower affinity to target molecule (Valadon and Scharff, 1996). In this assay, target molecules used for screening were immobilized on the plate, then the phage was added followed by anti-phage Abs.

I developed the streptavidin capture ELISA (Fig.2.1C) following the reverse ELISA format. In this assay, streptavidin as target molecule was immobilized on the plate to capture Fd phage displaying a biotin mimetic peptide from a mixed phage library. During each cycle of biopanning, the bsMAb ELISA signal mirrored the successive enrichment of the desired phage. We also showed that all three anti-phage M13 Abs (P106 bsMAb, P93 MAb and polyclonal Ab HRPO conjugates) were able to crossreact with fd phage with the same strength as that of M13 (Chapter 2.3.2.5). Thus, these Abs could be used to detect fd phage libraries by ELISA. I attempted to use this assay for monitoring the enrichment of target binding phage after each cycle of panning in the model biopanning study. This is usually determined by a plaque assay wherein the numbers of plaques in the eluates are compared with those from succeeding cycles of biopanning. An increase in the plaque number in successive cycles indicates enrichment of target binding phage. In my experiments, enrichment of biotin mimetic phages was observed by a continually increased ELISA signal with in each successive cycle of panning (Fig.4.3. curve a). This phage ELISA was not only a more rapid assay but also directly assayed the antigen specific phage unlike the general non-specific plaque assay.

The ELISA quantification of phage was used to determin the volume of PEG purified phage to be used for the next panning cycle and for comparative analysis. This is usually also performed by the plaque assay in the literature. In this study, the assay was performed on M13 sandwich ELISA (Fig. 2.1D) and data were compared with a standard curve in the linear range and with a conventional plaque assay. With good concordance between the ELISA and plaque method, I believe that this rapid and convenient phage sandwich bsMAb ELISA could replace the conventional plaque assay method.

Several groups have used anti-M13 polyclonal Abs to study and pan random peptides displayed on phage coat protein in *E.coli* (Stengele et al., 1990; MaCafferly et al., 1991) but polyclonal Abs may not be very suitable in experiments where phage preparations are contaminated with *E.coli* proteins giving high background and false positive. More recently an anti-phage monospecific MAb has been described (Bhardwaj et al., 1995) for the same purpose combined with a chemically crosslinked goat anti-mouse HRPO second antibody. Unfortunately, chemical procedures for enzyme labeling could result in aggregation, stickiness and inactivation of both MAbs and enzyme. If polyclonal second antibodies were used for developing a signal, they could cross-react with immobilized target molecules or coated impurities. Monospecific MAbs plus polyclonal second antibodies still exhibit a high background and reduce the sensitivity of detection. As shown in Fig.4.4 (inset), the backgrounds were high when using polyclonal anti-phage-HRPO and an indirect method with anti-M13 MAb and goat anti-mouse-HRPO when compared to anti-phage bsMAb-AP immune complexes. In the early stages of panning, when the number of target binding phages is relatively low, weak signals from the binding between desired phages and targets could be difficult to identify by MAbs or polyclonal Abs with any confidence if the background noise was high. In this situation, the advantages of the bsMAb in providing high sensitivity and low background are especially important. Table 1 and Fig.4.4. show ELISA results for determining the amount of streptavidin binding phage after the each amplification using the three different anti-phage antibody reagents. We could easily observe target binding phage using P106 after the first amplification. However, it was very difficult to judge if there was such phage using either the P93 or polyclonal Abs. The situation might be worse when the binding affinity between desired phage and target was lower since the signal could be very weak compared to background noise, even after second or third round of amplification.

To further test the sensitivity of our P106 bsMAb in detecting target binding phage in crude culture supernatant, I changed the conditions for the third panning and checked if the bsMAb based ELISA was able to accurately reflect the effects of these unfavorable conditions on enrichment of target binding phage. The conditions were

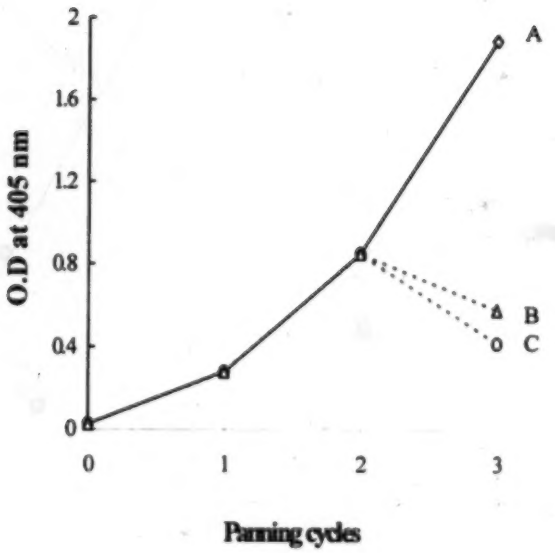


Fig.4.3 ELISA monitoring the of target binding phages in screening phage display peptide library: The crude culture supernatants from each amplification of eluted phage were checked on the streptavidin coated ELISA plate as described in Section 2.7.1. Curve A was the profile of a panning using a 100ug/mL streptavidin coated plate with a two step elution wherein phage from the second elution was used for amplification. Curve B employed a 5ug/mL streptavidin coated plate in the third panning. Curve C was performed by using a one step elution after third panning.

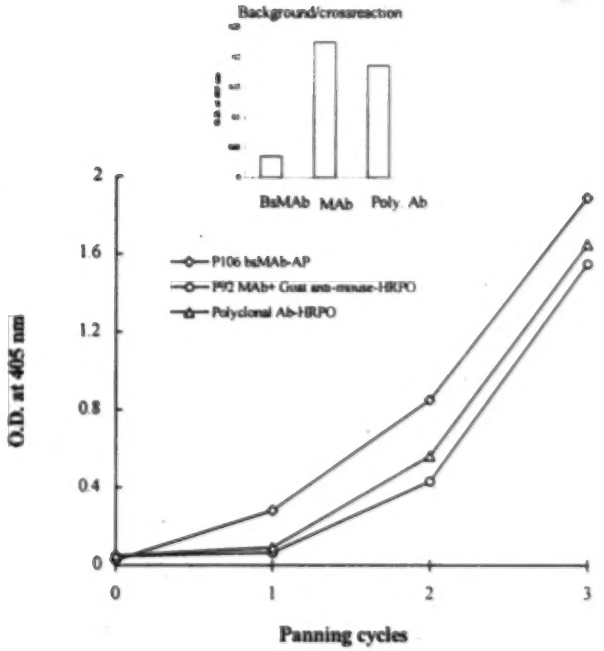


Fig.4.4. Comparison of three anti-phage antibodies in detecting target binding phages after each amplification cycle of phage library screening: The crude culture supernatants from each amplification of eluted phages were checked on the streptavidin coated plate as described in section 2.7.1. The bound phages were detected by P106 bsMAbs or P93 MAbs or polyclonal Abs. Each point was performed in duplicate and the value was calculated by subtracting signal in control wells. The crossreactions of three Abs with *E.coli* proteins were tested using cultured *E.coli* supernatant without phage infection (inset). Note that after the first cycle of panning, only the bsMAb signal can be discriminated from the background.

A. P106 bsMAb

Panning cycle	OD at assay well	OD at control well	Value
1	0.315	0.035	0.28
2	0.890	0.040	0.85
3	1.920	0.030	1.89

B. P92 MAb

Panning cycle	OD at assay well	OD at control well	Value
1	0.281	0.216	0.065
2	0.671	0.241	0.430
3	1.770	0.220	1.550

C. Polyclonal Ab

Panning cycle	OD at assay well	OD at control well	Value
1	0.277	0.187	0.09
2	0.755	0.195	0.56
3	1.826	0.176	1.65

Table 4.2 Primary data of the streptavidin capture ELISA after each amplification cycle: ELISA of M13 spiked library was performed on the streptavidin coated plate as described in section 4.2.4.1. *E.coli* culture supernatants from each amplification of phage library panning were added to assay wells and *E.coli* culture supernatant containing wild type phage were added to the control wells. P106 bsMAb or P92 MAb or polyclonal Ab were used as the probes to detect streptavidin binding phage. The data were used for plotting Fig.4.4.

changed by coating panning plate with 0.5 μg strepavidin instead of 10 μg and by a one step elution instead of a two step elution. In the two step elution, the phages eluted over a short incubation time with free biotin were discarded followed by a second elution with a longer incubation of the same free biotin. The phages from the second elution likely had higher affinity (higher number of biotin mimetic phage) and were taken into amplification in *E.coli.* The signals were apparently decreased (Fig.4.3. curves b and c) when the ELISA was performed using culture supernatant after the third amplification with either a one step elution or lower amount of streptavidin coating. This indicates that the eluates contained relatively less biotin mimetic phage compared to non-specific phage when taken to amplification in *E.coli.* From this result, phage ELISA could also be used to optimize biopanning conditions.

Although biopanning-amplification serves to enrich specific binding phage, not all selected phage represented the antigen specific clones. For example phage clones with reactivity to plastic (polystyrene) were isolated from a variety of previous screening experiments (Adey et al., 1995). So, it was common that tens of individual phage plaques had to be sequenced to ascertain a consensus sequence at the end of the panning procedure. This time consuming procedure could be simplified by a bsMAb based streptavidin capture ELISA before the extensive and laborious DNA sequencing. Based on the ELISA signal, these selected phage clones can be catalogued into groups that had different binding signals with the target molecule. Those phage clones with the desired strong reactivity would be selected and processed for DNA sequence analysis. After the fourth panning in our experiment, the eluates were plated into the culture dish at about 100 phage/plate. Fifty individual clones were checked by ELISA randomly. 80% of them presented high signald in our ELISA's (data omitted). Five clones were prepared for DNA sequence analysis. Two clones with high signal in the ELISA were shown to have the biotin mimetic peptide by comparing their DNA sequence with that of original biotin phages (Devlin et al., 1990) and three clones, which showed lower signal, proved to have an unrelated sequence. Fig.4.6 shows the ELISA signal of five clones with the corresponding DNA sequence of inserted peptide.

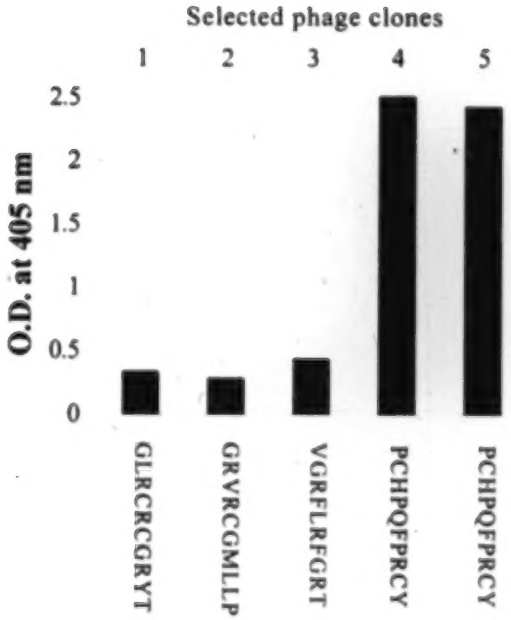


Fig.4.5. ELISA analysis of selected phage clones after fourth panning and corresponding peptide sequences: After fourth panning, eluted phage was plated at lower density. Fifty individual phage clones were picked up and propagated in 3 mL *E.coli* culture. A 100 µl aliquot of culture was used for streptavidin capture ELISA. The two clones with high signal and three with low signal were chosen for DNA sequencing.

4.3.3. BsMAb based affinity method for purificaton of viruses

The conventional method of virus purification using ultracentrifugation frequently results in damaged particles with low levels of biological activity, and thus it is unsuitable for preparing samples for high-resolution techniques such as neutron scattering, X-ray scattering in solution, and X-ray crystallography (Crook et al., 1985). Moreover, significant biohazard could be generated by aerosol or by an instrument failure when preparing pathogenic viruses or subunits derived from them. Antibody immobilized agarose bead columns have been used as a general approach for the purification of viruses (Ruoslahti, 1976). This method can purify viruses with a high specificity in a relatively simple and safe manner. However, harsh conditions have to be used for the elution of viruses from the immune complexes. Damaged virus particles and reduced biological activity are some of problems associated with this kind of immunoaffinity purification of viruses.

Another novel application of the bsMAb developed in this thesis is in the development of simple and safe methods of virus purification or clearance. We exploited the Mimetic affinity column to demonstrate the feasibility of this approach. Mimetic blue AP A6XL adsorbent consists of a blue chromophore linked to a functional phosphoric acid group and demonstrated a high affinity with calf intestinal alkaline phosphatase (Lindner et al., 1989). As described in Chapter 3.3.3, P106 bsMAbs can be co-purified with AP as immune complexes because one paratope has affinity to AP. Similarly, it is possible that P106 bsMAbs can bind phage with the second anti-phage paratope when they are bound to AP on the mimetic blue affinity absorbents. If bsMAb could bridge AP and phage together on the mimetic blue affinity matrix, this should allow removal of the whole virus-bsMAb complex from other soluble proteins. Since the immune complexes can be eluted with either phosphate buffer under mild condition or with excess AP, there will be no risk of physical damage to the viruses.

Firstly, purification was performed on the AP coated surface of a microtiter plate as described in section 4.2.5.1. This simple experiment was for demonstrating if bsMAb could bridge solid phase AP and phage in solution on the surface of the microtiter well

and showing that such immune complexes can be eluted off the surface by excess AP. Since it is likely that even after blocking, some phage can bind to the plastic surface nonspecifically, the same experiment was performed on a non-AP-coated surface as background. The number of specifically bound and eluted phages was calculated by the number of phages eluted from the AP coated well minus the background. The eluted phages were quantified by a plaque assay to determine purification yield. The results in table 4.2A shows that the specific binding and elution of phage were increased with increasing the number of input phage until saturation was reached presumably due to the limited number of bsMAbs on the surface. The maximum purification yield was only 0.6% by the microplate method.

Hence, I further tested if larger amounts of phage could be co-purified with bsMAb-AP immune complexes on the Mimetic blue affinity absorbent. To ensure that the samples (AP, bsMAbs and phage) could adequately contact the absorbent, a batch mode was used for purification wherein the samples were incubated with absorbent in a flask with gentle shaking and unbound fractions were washed. Table 4.2B shows the data from a group of purification experiments. The results, although demonstrating the principle of affinity purification, had a poor yield. This was likely due to insufficient binding sites available for phage on the surface of the matrix. According to the manufacturer's specification, the bead pore size of Mimetic blue AP A6XL adsorbent was optimal for 8 x 10^5 Da molecular weight of globular proteins. The bead pore size could be too small for phage to enter the inside of the bead because the M13 phage has a molecular weight of 1.2 x 10^7 Da (Henry et al., 1969). It was assumed that most of the phosphonate ligands were inside the bead, and only small number of them was on the surface of the bead. Thus, the potential surface phage binding sites generated for AP-bsMAb immune complexes would be very limited. However, the ability of anti-phage/anti-AP bsMAbs to purify phage was demonstrated on the surface of microtiter plate with a slightly better yield. Further optimization of viral purification or removal could be done by developing non-porous Mimetic blue affinity supports wherein the ligands are primarily on the surface of such a matrix for a better yield. Such optimized techniques would have applications in producing virus free pharmaceuticals. Alternatively, these procedures

A

Input phage	Eluted phage (bsMAb plate)	Eluted phage (control plate)	yield %
10^6	$5 X 10^4$	100	0.5%
10^7	$6 X 10^5$	500	0.6%
10^8	$4 X 10^6$	500	0.4%
10^9	$6 X 10^7$	600	0.6%
10^{10}	$4 X10^7$	600	0.04%

B

Input phage	Eluted phage (bsMAb column)	Eluted phage (control column)	yield %
$5 X 10^6$	$7 x 10^4$	10^3	0.14%
$5 X 10^7$	10^5	$4 x 10^3$	0.2%
$5 X 10^8$	$4 x 10^6$	10^4	0.8%
$5 X 10^9$	10^7	$3 X10^5$	0.2%
$5 X10^{10}$	$4 x 10^6$	$2 X 10^5$	0.1%

Table 4.3 A. BsMAb affinity purification of M13 phage on microtiter plate: BsMAb and M13 phage was incubated with AP coated microtiter plate in 2 steps and the wash step was performed between two steps. Bound phage was eluted by adding free AP as described in section 4.6.1. B. BsMAb affinity purification of M13 phage on Mimetic blue column: AP, bsMAb and M13 phage were incubated with Mimetic blue AP A6XL absorbent in 3 steps and wash steps were performed after each incubations. Bound phage were eluted with phosphate buffer as described in section 4.6.2.

could be used in the purification of medically important viruses to prepare attenuated vaccines, avoiding complex and biohazardous purification steps.

4.3.4.Conclusion

The bsMAb P106 based ELISA, as reliable tool, considerably reduced the time in panning desired phage from a phage peptide library and hence could potentially assist in accelerating the discovery of proteins with new function and putative peptide drugs. The enzyme amplified virus ELISA using P106-AP immune complexes proved to be extremely sensitive in detecting virus particles. The principle of this assay could be used in constructing new assays for clinical diagnostics and monitoring of viral or bacterial infection and treatment. Ultrasensitive assays measuring infective particles or their antigens to mirror the active disease state could perform better than a host of current clinical assays measuring specific antibodies to those antigens which only serve to document a previous history of infection. Lastly, bsMAb based affinity methods of purifying viruses has the potential to overcome the disadvantages of traditional methods and purify virus with a high specificity and purity using mild conditions.

Chapter 5. Summary, Conclusions and Future Work

This thesis presented the experimental studies on the developments of anti-phage M13/anti-AP bsMAbs and anti-phage M13/anti-HRPO bsMAbs; the applications of anti-phage M13/anti-AP bsMAbs in viral detection, phage display technology and viral purification.

Firstly, a hybridoma producing anti-phage M13 antibody was generated by fusing myeloma SP2/0 cells with the splenocytes collected from a mouse immunized with the $CsCl_2$ gradient purified phage M13 particles. The best hybridoma cell line P93.11 secreting anti-phage MAb was established and characterized. The results indicate that the P93.11 belongs to the IgG2a subclass. It reacts to phage particle in ELISA with the highest avidity compared with other three clones but does not react with denatured phage coat protein on a Western blot. The P93.11 MAb also bound to fd phage with comparable strength to M13 phages and but did not crossreact to *E.coli* proteins. The P93.11 MAb was produced in the mouse ascites and purified by DE-52 ion exchange chromatography.

The quadromas producing bsMAbs anti-phage M13/anti-AP (P106.5B) were developed by fusion of hybridomas P93 with P92 (anti-AP). The hybridoma fusion partners were labeled with two different fluorescence markers for 30 min before electrofusion and double fluorescent quadromas were selected by FACS. Five quadromas were screened and recloned. Among them, P106.5B was the best, presenting a high ELISA signal during one and half months of continuous culture. Mouse ascites with P106.5B activity were collected from mice injected with the quadromas for further purification.

The triomas producing bsMAbs anti-phage M13/anti-HRPO (P107) were developed by PEG-fusion of hybridoma YP4 with the splenocytes from a mouse immunized with $CsCl_2$ gradient purified phage particles. YP4 is a rat hybridoma secreting anti-HRPO MAb IgG1 and back-selected for HAT sensitivity developed previously in our Lab. A total 24 bsMAb secreting triomas were selected by HAT medium.

A Mimetic blue A6XL affinity absorbent which was originally designed for the purification of AP from calf intestinal was employed for purification of anti-phage

M13/anti-AP bsMAb P106.5B from anti-phage monospecific MAb P93.11 secreted by the same quadroma. P106 bsMAbs bound to AP pre-absorbed on the affinity matrix after the crude bsMAb sample was dialyzed to eliminate phosphate, and were subsequently eluted as bsMAb-AP immune complexes. The results from both ELISA and Western blot analysis proved the contaminants such as monospecific anti-phage MAb and other impurities were efficiently eliminated as unbound materials. The elution of bsMAb-AP immune complexes was performed under a very mild condition wherein 10 mM phosphate in 10 mM tricine buffer was used. Neither bsMAb nor AP are likely to damaged under this condition.

The uniform reproducible enzyme binding to the bsMAb results in superior immunoconjugate in comparison with chemical procedures which result in random inter- and intra- molecular cross-links and mutiprotein aggregates. In a comparative study, the purified P106.5B-AP immune complexes showed 10 times higher sensitivity in detecting phage M13 than both HRPO conjugated anti-phage M13 polyclonal antibody and P93 anti-phage M13 monospecific MAb plus HRPO conjugated second antibody. This highly sensitive immune probe allowed us to further develop a bsMAb based immunoassay for virus detection and for screening a phage display library. It is important to note that this affinity purification method also co-purifies the monospecific anti-AP antibody along with the bsMAb. However, the later species does not interfere with immunoassays and is washed away prior to substrate addition.

An ultrasensitive immunoassay for virus was developed using phage M13 as a model virus. The viruses were captured by solid phase P93.11 anti-phage MAb and P106.5B bsMAb-AP immune complexes were used to detect the captured viruses in the solid phase. The unique design of this assay employed AP bound to one paratope of bsMAb coupled with a cyclic enzymatic amplification for enhanced sensitivity. The practical sensitivity of the assay was 100 phage particles (1000 pfu/ml) which approximately equal to 2.3 pg (23pg/ml). A 1000-fold increase in sensitivity was achieved compared with conventional signal development method using pNpp.

A biopanning experiment was carried out for selecting phages displaying biotin mimetic peptide from a mixed phage peptide library. The P106.5B bsMAb based ELISA

was used to detect target binding phages, monitor enrichment of target binding phage and quantify phages after each panning cycle. This resulted in rapid selection of the 'biotin mimetic peptide sequence' from millions of other phages in only 6 days. The selected phage clones with high ELISA signal were proved by peptide sequence analysis as having the same peptide sequence as that of the original biotin phage.

Another novel application of the bsMAb demonstrated in this thesis is in the development of simple and safe method of virus purification or clearance. In a microtiter plate method, P106 anti-phage M13/anti-AP bsMAbs were immobilized on AP coated solid phase to capture phage particles from a crude phage preparation. Other protein contaminants were removed by a wash step. The bound viruses were eluted off by adding excess AP. A similar principle was used to purify phage using the Mimetic Blue absorbent. The virus were complexed with bsMAb-AP on Mimetic Blue column to resolve from other soluble proteins, then the viruses were eluted from column by 10 mM phosphate buffer as virus-bsMAb-AP complexes. Elution condition is mild without any risk of physical damage to the viruses. The number of eluted phage increased with input phage with a slightly better consistency in microtitre plates. The yields from Mimetic Blue column method were poor which was possibly caused by inefficient binding sites on the Mimetic blue absorbent due to the smaller pore size compared with the larger phage particles.

In conclusion, (1) Electrofusion with FACS selection is a fast method to produce bsMAb secreting quadromas for diagnostic applications. BsMAbs can also be produced by developing a trioma, a slightly different approach than quadroma production. (2) By exploiting Mimetic blue A6XL affinity chromatography, P106 bsMAb-AP immune complexes can be purified in a single step with high specific activity. The purified bsMAb-AP immune complexes can be used directly in the enzyme-based immunoassay with a higher sensitivity than both anti-phage M13 MAb plus HRPO conjugated second antibody and HRPO conjugated anti-phage polyclonal AP. (3) An ultrasensitive immunoassay for virus was developed using P106 bsMAb-AP immune complexes linked with a cyclic enzyme amplification technique to increase signal intensity. This achieved a 1000 fold increase in sensitivity over conventional assays wherein pNpp was used as the

substrate for signal development. The principle of this assay could be used in constructing new assays for clinical diagnostics and monitoring of viral or bacterial infection and treatment. (4) The P106 bsMAb based ELISA, as a reliable tool, considerably reduced the time in panning desired phages from a phage peptide library and hence could assist in accelerating the discovery of proteins with new function and putative peptide drugs. (5) The ability of bsMAb for purifying virus has been shown. The results indicate that bsMAb based affinity method has the potential to overcome the disadvantages of traditional method and purify viruses with high specificity and fully conserved biology activity. However, this application needs to be improved further.

Future studies to be accomplished include (1) characterizing our anti-phage M13/anti-HRPO trioma and investigate the applications of anti-phage M13/anti-HRPO bsMAb. (2) further improving sensitivity of viral detection by using polyalkaline phosphatase instead of native monomeric AP or use induce fluorescence detection of viral bound AP to achieve detection of a single viral particle. (3) optimizing viral purification or removal by developing a non-porous Mimetic Blue affinity support.

Reference

Affleck, K. and Embleton, MJ. (1992) Monoclonal antibody targeting of methotrexate (MTX) against MTX-resistant tumor cell lines. *Br. J. Canc.*, 65:838.

Adey, NB., Mataragnon, AH., Rider, JE., Carter, JM. and Kay, BK. (1995) Characterization of phage that bind plastic from phage displayed random peptide library. *Gene,* 156:27.

Allard, WJ., Moran, CA., Nagel, E., Collins, G. and Largen MT. (1992) Antigen bindign properties of highly purified bispecific antibodies. *Mol. Immunol.*, 29:1219.

Arase, Y., Kumada, H., Chayma, K., Naoya, M., Tsubota, A., Koida, I., Kobayashi, M., Suzuki, Y., Ikeda, K. and Saitoh, S. (1995) Usefulness of HCV-RNA counts by the method of HCV-bDNA probe in interferon retreatment for patients with HCV-RNA positive chronic hepatitis C. *Int. Hepatol. Conmuni.*, 4:19.

Auriol. J., Guesdon, JL., Mazie, JC. and Nato, F. (1994) Development of a bispecific monoclonal antibodies for use in moecular hybridization. *J. Immunol. M.*, 169:123.

Bass, S., Greene, R. and Wells, JA. (1990) Hormone phage: an enrichment method for variant proteins with altered binding properties. *Proteins,* 8:309.

Baker, RM., Brunette, DM., Thompson, LH., Siminovitch, L. and Till, J.E. (1974) Ouabain-resistant mutants of mouse and hamster cell in culture. *Cell,* 1:9

Barbas, CF., Jr Crowe, JE., Cababa, D., Jones, TM., Zebedee, SL., Murphy, BR., Chanock, RM. and Burton, DR. (1992) Human monoclonal Fab fragments derived from a combinatorial library bind to respiratory syncytial virus F glycoprotein and neutralize infectivity. *Proc. Natl. Acad. Sci. USA*, 89:10164.

Barbara, D. and Klaus, RN. (1996) Applying viral load measurements to HIV care. *HIV News,* 21:103.

Berkowitz, S. and Day, L. (1976) Mass, Length, composition and structure of the filamentous bacterial virus fd. *J. Mol. Biol.*, 102: 531.

Bates D.L.(1987) Enzyme amplification in diagnostics. *TIBTECH.* 5:204.

Bhardwaj, D., Singh, SS., Abrol, S. and Chaudhary, VK. (1995) Monoclonal antibodies against a minor and the major coat proteins of filamentous phage M13: their application in phage display. *J.Immunol. M.*, 179:165.

Bonardi, MA., Bell, A., French, RR., Gromo, G., Hamblin, T., Modena, D., Tutt, AL. and Glennie, MJ. (1992) Initial experience in targeting human lymphoma with a combination of bispecific antibody and saporin. *Int. J. Cancer-Suppl.*, 7:73.

Bonardi, MA., French, RR., Amlot. P., Gromo, G., Hamblin, T., Modena, D. and Glennie, MJ. (1993) Delivery of saporin to human B-cell lymphoma using bispecific antibody: targeting via CD22 but not CD19, CD37, or immunoglobulin results in efficient killing. *Cancer Res.*, 53:3015.

Boni, J., Opravil, M., Tomasik, Z., Rothen, M., Bisset, L., Grob, P.J., Luthy, R., and Schupbach, J. (1997) Simple monitoring of antiretroviral therapy with a signal-amplification-boosted HIV-1 p24 antigen assay with heat-denatured plasma. *AIDS*. 11: F47.

Brackke, M. K.(1951) 'Density Gradient Centrifugation; A New Separation Technique", *J. Amer Chem Soc*. 73:1847.

Brackke, M.K. (1967) "Density gradient centrifugation" in Methods in Virology, Vol. 2 K. Maramorosch and H. Koprowski, eds. New York: Academic Press, pp93.

Boeke, J. (1981) One and two codon insertion mutants of bacteriophage f1. *Mol. Gen. Genet.* 181:288

Cao, Y. and Suresh, MR. (1998) Enhanced tumor specific drug delivery by pretargeting bispecific MAb and biotin-PEGylated liposome. *Bioconj.Chem*., in press.

Cannon, LE., Ladner, RC. and McCoy, D. (1996) Phage display technology. *IVD technology*, Nov/Dec,22.

Chao, J., DeBiasio, R., Zhu, Z., Giuliano, KA. and Schmidt, BF. (1996) Immunofluorescence signal amplification by the enzyme-catalyzed deposition of a fluorescent reportor substrate (CARD).*Cytometry.*, 23:48.

Christian, RB., Zuckermann, RN, Kerr, JM., Wang, L. and Malcome, BA. (1992) Simplified methods for construction, assessment and rapid screening of peptide libraries in bacteriophage. *J.Mol. Biol*. 227:711.

Chatal, JF., Faivre-Chauvet, A., Bardies, M., Peltier, P., Gautherot, E. and Barbet, J. (1995) Bifunctional antibodies for radiioimmunotheraphy. *Hybridomas,* 14:125.

Chang, DC., Saunders, JA., Chassy, BM. and Sower, AE. (1992) Overview of electroporation and electrofusion. In: D.C. Chang, J.A. Saunders, B.M., Chassy, A.E. Sowers (Eds), Guide to electroporation and electrofusion. Academic Press, San Diego, CA.

Chetanneau, A., Barbet, J., Peltier, P., Le Doussal, JM., Gruaz-Guyon, A., Bernard, AM., Resche, I., Rouvier, E., Bourguet, P., Delaage, M., and et al. (1994) Pretargetted imaging of colorectal cancer recurrences using an 111 In-labeled bivalent hapten and a bispecific antibody conjugated. *Nucl. Med. Communications*, 15:972.

Chervonsky, AV., Faerman, AL., Kazarov, AR. and Gussev, AI. (1988) A simple metabolic system for selection of hyrid hybridomas (tetradomas) producing bispecific monoclonal antibodies. *Mol. Immunol.*, 25:913.

Christopoulos T.K. and Diamandis E.P. (1996) Immunoassay configurations. In Diamandis E.P., Christopoulos T.K. (Eds), Immunoassy. p227. Academic Press Inc., San Diago, CA.

Clark, PMS. and Price, CP. (1986) Enzyme-amplified immunoassay: a new ultrasensitive assay of thyrotropin evaluated. *Clin. Chem.*, 32:88.

Cook, AG. and Wood, PJ. (1994) Chemical synthesis of bispecific monoclonal antibodies: potential advantages in immunoassay systems. *J. Immunol. M.*, 171:227.

Cook, DB. and Self, CH. (1993) Determination of one thousandth of an attomole (1 zeptomole) of alkaline phosphatase: Application in an immunoassay of proinsulin. *Clin. Chem.* 39: 965.

Cobbold, SP. and Waldmann, H. (1984). Therapeutic potential of monovalent monoclonal antibodies. *Nature* 308:460.

Coloma, M.J. and Morrison, S.L. (1996) Design and production of novel tetravalent bispecific antibodies. *Nat. Biotechnol.*, 15:159.

Corvalan, JR., Smith, W., Gore, VA. and Brandon, DR. (1987) Specific in vitro and vivo drug localisation to tumor cells using a hybrid-hybrid monoclonal antibody recognising both carcinoembryonic antigen (CEA) and vinca alkaloids. *Cancer Immunol. Immunother.*, 24:133.

Craig, DB; Arriaga, E; Wong, JCY; Lu, H and Dovichi, NJ. (1996) Study on single alkaline phosphatase molecules: Reaction rate and activation energy of a reaction catalyzed by a single molecule and effect of thermal denaturation-the death of an enzyme. *J.Am. Chem.Soc.* 118:5245.

Crooks, AJ., Lee, JM. and Stephenson., JR. (1985) " The purification of alphavirus virions and subviral particles using ultrafiltration and gel exclution chromatography. *Arch.Biochem. Biophys.*, 45:275.

Davis, GL. (1994) Precdiction of response to interferon treatment of chronic hepatitis C. *J. Hepatol.*, 21:1.

Devlin, JJ., Panganiban, LC. and Devlin, PE. (1990) Random peptide libraries: a source of specific protein binding. *Science*, 249: 405.

Dente, L., Cesareni, G., Micheli, G., Felici, F., Folgori, A., Luzzago, A., Monaci, P., Nicosia, A., and Delmastro, P., (1994) Monoclonal antibodies that recognise filamentous phage: tools for phage display technology. *Gene*, 148: 7.

Demanet, C., Brissinck, J., Van Mechelen, M., Leo, O. and Thielemans, K. (1991) Treatment of murine B cell lymphoma with bispecific monoclonal antibodies (anti-idiotype x anti-CD3). *J Immunol.*, 147: 1091.

Dedman, JR., Kaetzel, MA., Chan, HC., Nelson, DJ. and Jamieson, GA. (1993). Selection of target biological modifiers from a bacteriophage library of random peptides: The identification of novel calmodulin regulatory peptides. *J. Biol. Chem.*, 268: 23025.

De Lau, WB., Van Loon, AE., Heije, K., Valerio, D. and Bast, BJ. (1989) Production of hybrid hybridomas based on HAT(s)-neomycin(r) double mutants. *J. Immunol. M.*, 117:1.

Dennis, MS. and Lazarus, RA. (1994). Kunitz domain inhibitors of tissue foctor factor VIIa. I. Potent inhibitors selected from libraries by phage display. *J.biol.chem.*, 269: 2129.

Dhahir, F., Cook, DB., Self, CH. (1992) An amplified enzyme-linked immunoassay for human proinsulin. *Clin Chem.*, 38:227.

Diamandis, EP., Yu, H. and Sutherlans, DJ. (1994) Detection of prostate-specific antigen immunoreactivity in breast Cancer. Res. *Trat.*, 32:291.

Doorbar, J. and Winter, G. (1994). Isolation of peptide antagonist to the thrombin receptor using phage display. *J.Mol.Biol.*, 244:361.

Dwyer, DE., Adelstein, S., Cunningham, AL. and Merigan, TC. (1996). The laboratory in managing HIV infection. *Med. J. Australia.*, 164:301.

Eshhar, Z., Waks, T., Gross, G. and Schindler, DG. (1993) Specific activation and targeting of cytotoxic lymphocytes through chimeric single chains consising of antibody-binding domains and the gamma or zeta subunits of the immunoglobulin and T-cell receptors. *Proc. Natl. Acad. Sci. USA*, 90: 720.

Ferrini, S., Prigione, I., Miotti, S., Ciccone, E., Cantoni, C., Chen, Q., Colnaghi, MI.and Moretta, L. (1991) Bispecific monoclonal antibodies directed to CD16 and to a tumor-associated antigen induce target-cell lysis by resting NK cells and by a subset of NK clones. *Int. J. Cancer*, 48:227.

French, RR., Hamblin, TJ., Bell, AJ., Tutt, AL., and Glennie, MJ.(1995) Treatment of B-cell lymphomas with combination of bispecific antibodies and saporin. *Lancet.*, 346:223.

Gonik, B., Seibel, M., Berkowitz, A. Woodin, MB. and Mills, K. (1991) Comparison of two enzyme-linked immunosorbent assay for detection of herpes simplex virus antigen. *J. Clin. Microbiol.* 29:436

Glennie, MJ., McBride, HM., Worth, AT. and Stevenson, GT. (1987) Preparation and performance of bispecific $F(ab'\gamma)_2$ antibody containing thioether-linked $Fab'\gamma$ fragments. *J. Immunol.* 139: 2367.

Gosling, J.P. (1990) A decade of development in immunoassay methodology. *Clin. Chem.*, 36:1408.

Gorog, G., Gandolfi, A., Paradisi, G., Rolleri, E., Klasen, E., Dessi, V., Strom, R. and Celada, F. (1989) Use of bispecific hybrid antibodies for the development of a homogeneous enzyme immunoassay. *J. Immunol. M.*, 123:131.

Goldsmith, M. and Konigsberg, W. (1977). Adsorption protein of the bacteriophage fd: isolation, molecular properties, and location in the virus. *Biochmistry*, 16:2686.

Hammerling, U., Aoki, T., De Harven, E., Boyse, E.A. and Old, LJ. (1968). Use of hybrid antibodies with anti-gama G and anti-ferritin specificities in locating cell surface antignes by electron microscopy. *J. Experi. Med.*, 128:1461.

Hawkins, A., Davidson, F. and Simmonds, P. (1997) Comparison of plasma virus loads among individuals infected with hepatitis C virus (HCV) genotypes 1, 2, and 3 by quantiples HCV RNA assay versions 1 and 2, Roche monitor assay and an in-house limiting dilution method. *J. Clin. Microbio.*, 35:187.

Henry, T. and Pratt, D. (1969). The proteins of bacteriophage M13. *Proc. Natl. Acad. Sci.USA*, 62: 800.

Holliger, P., Prospero, T. and Winter, G. (1993) "diabodies': small bivalent bispecific antibody fragments. *Procee. Nat. Acade Sci. USA*, 90:6444.

Ho, DD. (1996) Viral count in HIV infection. *Science*, 272:1124.

Hsiung, GD. (1994) Enzyme-linked immunosorbent assay for antigen detection and antibody determination. In Hsiung GD. Fong CK. and Landry ML.'s Diagnostic virology. p 62-64. Yale University Press. New Haven and London.

Hurwitz, E., Levy, R., Maron, R., Wilchek, M., Arnon, R. and Sela, M. (1975) The covalent binding of daunomycin and adriamycin to antibodies, with retention of both drug and antibody activities. *Cancer Res.*, 35:1175.

Ishikawa, E. (1996) Labeling of antibodies and antigens. In Diamandis E.P., Christopoulos T.K. (Eds) Immunoassy. P191-204. Academic Press Inc, San Diago, CA.

Kay, BK. Winter, J. and McCafferty, J. (1996) In Phage display of peptides and proteins. A library manual. pp227. Academic Press Inc, San Diego, CA.

Kappes, JC., Saag, MS., Shaw, GM., Hahn, BH., Chopra, P., Chen, S., Emini, EA., McFarland, R. and Yang, LC. (1995) Assessment of antiretroviral therapy by plasma viral load test: standard and ICD HIV-1 p24 antigen and viral RNA (OC-PCR) assays compared. *J. Acquir. Immune. Defic. Syndr. Hum. Retrovirol.*, 10:139.

Karawajew, KL., Micheel, B., Behrsing, O. and Gaestel, M. (1987). Bispecific antibody-producing hybrid hybridomas selected by a fluorescence activated cell sortor. *J. Immunol. M., 96:256.*

Kohler, G. and Milstein. (1975) Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature*, 256:495.

Kontsekova, E., Novak, M., Macikova, I. and Kontsel, P. (1991). One-step method for establishing 8-azaguanine-resistant hybridomas suitable for the preparation of triomas. *J.Immunol. M.*, 145:247.

Kranz, DM., Gruber, M. and Wilson, ER. (1995) properties of bispecific single chain antibodies expressed in Escherichia coli., *J. Hematotherapy*, 4:403.

Kuppen, PJ., Eggermont, AM., Smits, KM., Van Eendendenburg, JD., Lazeroms, SP., Van De Velde, CJ. and Fleuren, GJ. (1993) The development and purification of a bispecific antibody for lymphokine-activated killer cell targeting against the rat colon carcinoma CC531. *Cancer Immuno. Immuothe.*, 36:403.

Kreutz, FT. and Suresh, MR (1995) Bispecific monoclonal anti-CA125 x anti-peroxidase antibodies in the measurement of the ovarian carcinoma antigen. *J. Tumor Marker Oncol.*, 10:45.

Kreutz, FT. and Suresh, MR. (1997) Novel bispecific immunoprobe for rapid and sensitive detection of prostate-specific antigen. *Clin. Chem.*, 43:4.

Kreutz, FT., Xu, DZ. and Suresh, MR. (1998). A new method to generate quadromas by electrofusion and FACS sorting. *Hybridoma*,. 17:267.

Littlefield, LW. (1964) Cold Spr.Harb, *Symp. Quant. Biol.* 29:161.

Lin,TC., Webste, RE. and Konigsberg, W. (1980) Isolation and charaterization of the C and D proteins coded by gene IX and gene VI in the filamentous bacteriophage f1 and fd. *J.Biol. Chem.*, 255:10331.

Lindner, NM., Jeffcoat, R. and Lowe, CR. (1989) Design and applications of biomimetic anthraquinone dyes, purification of calf intestinal alkaline phosphatase with immobilized terminal ring analogues of C.I. reactive blue2. *J. Chromatogr.*, 473:227.

Lowman, HB. and wells, JA. (1993) Affinity maturation of human growth hormone by monovalent phage display. *J. Mol.Biol.*, 234:564.

Markland, W., Roberts, BL., Saxena, MJ., Guterman, SK. and Ladner, RO. (1991) Disign, construction and function of a multicopy display vector using fusions to the major coat protein of bacteriophage M13. *Gene,* 109:13.

Markoulatos, P., Fountoucidou, P., Marinakis, G., Krikelis, V., Spyrou, N., Vamvakopoulos, N. and Moncany, ML. (1997) Clear detection and typing of herpes simplex virus types 1 and 2 by an indirect ELISA assay: comparison with three different combined methods-capture ELISA, restriction enzymes, and polymerase chain reaction. *J. Clin. Lab. Anal.*, 11:146.

Marx, J.(1982) Monoclonal antibodies in cancer. *Science,* 216:283

Marks, JD., Hoogenboom, H., Bannert, T., McCafferty, J., Griffiths, AD. and Winter, G. (1991) By-passing immunisation: human antibodies from V-gene libraries displayed on phage. *J. Mol. Biol.,* 222: 581.

Marvin, D., Pigram, W., Wiseman, R., Wachtel, E. and Marvi, F. (1974) Filamentous bacterial viruses. XII. Molecule architecture of the class I (fd, If1, Ike) virion. *J. Mol. Biol.*, 88:581.

McCafferty, J., Griffiths, AD., Winter, G. and Chiswell, DJ. (1990) Phage antibodies: filamentous phage displaying antibody variable domains. *Nature*, 348: 552.

McCafferty, J., Jackson, RH. and Chiswell, DJ.(1991) Phage-enzymes: expression and affinity chromatography of functional alkaline phosphatase on the surface of bacteriophage. *Prot. Eng.*, 4:955

McCafferty, J., FitzGerald, K.J., Earnshaw, J., Chiswell, D.J., Link, J., Smith,R. and Kenten, J. (1994) Selection and rapid purification of murine antibody fragment that bind a transition-state analog by phage display. *Appl. Biochem. Biotehnol.*, 47:157.

Milstein, C. and Cuello, AC. (1983) Hybrid hybridomas and their use in immunohistochemistry. *Nature*, 305:537.

Mellors, JW., Rinaldo, Jr. CR., Gupta, P., Roseanne, M., White, RM., Todd, JA. and Kingsley, LA. (1996) Prognosis in HIV-1 infection predicted by the quantity of viruse in plasma. *Science*, 272: 167.

Mellors, JW., Munoz, A., Giorgi, JV., Joseph, B., Charles, R. and Rinaldo, Jr. (1997) Plasma viral load and $CD4^+$ lymphocytes as prognostic marker. *Ann. Intern. Med.*, 126: 946.

Mita, E., Hayashi, N., Hagiwara, H., Ueda, K., Kanazawa, Y., Kanazawa, A., Fusamoto, H. and Kamads, T. (1994) Predicting interferon therapy efficacy from hepatitis C virus genotype and RNA titer. *Dig. Dis. Sci.*, 39:1273.

Morein, B., Helenius, A., Simons, K., Petterson, R., AK; Kaariainen, L. and Schirrmacher, V.(1978) *Nature*, 276:715.

Morrison, SL. and Oi, VT. (1989) Genetically engineered antibody molecules. *Adv. Immunol.*, 44:65.

Mok, J., DeRossi, A., Ades, A., Giaquinto, C., Grosch-Worner, I. and Peckham, C. (1987) Infants born to mothers seropositive for human immunodeficiency virus. *Lancet*, I:1164.

Nauta, WJ. and Gydax, PA. (1951) Protein purification. New york: Academic press, pp405.

Newman, J., Swinney, H. and Day, L. (1977) Hydrodynamic properties and structure of fd virus. *J. Mol. Biol.*, 116:593.

Nisonoff, A. and Rivers, MM. (1961) Recombination of a mixture of univalent antibody fragments of different specificity. *Arch. Biochem. Biophys.*, 93: 460.

Nissim, A., Hoogenboom, HR., Tomlinson, LM.., Flynn, G., Midgley, C., Lane, D. and Winter, G.(1994) Antibody fragments from a 'single pot' phage display library as immunochemical reagents. *The EMBO Journal*, 13:692.

Nolan, O. and O'Kennedy, R. (1990) Bifunctional antibodies: concept, production and applications. *Biochim. Biophys. Acta.*, 1040:1.

Nozaki, Y. Reynolds, J. and Tanford, C.(1978) Conformational states of a hydrophobic protein. The coat protein of fd bacteriophage. *Biochemistry* 17:1239.

Opella, SJ. Cross, J. Di Verdi and Sturm, C. (1980) Nuclear magnetic resonace of the filamentous bacteriophage fd. *Biophys. J.* 32:531.

Parmley, SF. and Smith, GP. (1988) Antibody-selectable filamentous fd phage vectors: affinitypurification of target genes.*Gene*, 73:305.

Pere, P., Hoffman, RW., Shaw, S. and Pere, P. (1985) Specific targeting of cytotoxic T cells by anti-T3 linked to anti-target cell antibody. *Nature*, 316:354.

Piatak, M. Jr., Saag, MS., Yang, SC., Clark, JC., Kappes, KC., Luk, BH., Hahn, GM. and Shaw, JD. (1993) High level of HIV-1 in plasma during all stages of infection determined by competitive PCR. *Science*, 259:1749.

Porstmann, B., Porstmann, T., Nugel, E. and Evers, U. (1985) Which of the commonly used marker enzymes gives best result in colorimetric and fluorimetric enzyme immunoassays: horseradish peroxidase, alkaline phosphatase or ß-galactosidase. *J. Immuno. M.*, 79:27.

Porstmann, T. and Kiessig, ST. (1992) Enzyme immunoassay techniques. A overview. *J. Immunol*. M., 150:5.

Poloni, F., Castagna M., Felici, F. and Cianfriglia M. (1997) A new immunohistochemical methodology for the specific detection of MDR1-P-Glycoprotein in human tissues based on phage-displayed peptides mimicking the MM4.17 epitope. *Biol. Chem.*, 378:503.

Polin, RA. (1984) Monoclonal antibodies against microorganisms. *Eur. J. Clin. Microbiol.*, 3:387.

Renner, C., Jung, W., Sahin, U., Denfeld, R.. Pohl, C., Trumper, L., Hartmann, F., Diehl, V. and van Lier, R. (1994) Cure of xenografted human tumors by bispecifc monoclonal antibodies and human T cells. *Science*, 264: 833.

Reiken, SR., Van Wie, BJ. and Sutisna, H. (1996) Bispecific antibody modification of nicotinic acetylcholine receptors for biosensing. *Biosens. Bioelectron.*, 11:91.

Reddy, VS., and Ford, CH. (1993) Production of hybrids secreting bispecific antibodies recognising CEA and doxorubicin. *Anticancer Res.*, 13:2077.

Roberts, BL., Markland, W., Ley, AC., Kent, RB., White, DW., Guterman, SK. and Ladner, RC. (1992) *Proc. Natl. Acad. Sci. USA* 89:2429.

Ruoslahti, E. (1976) "Immunoadsorbents in protein purification" *Stand. J. Immunol. Suppl.3.*

Sambrook, J., Fritsch, EF. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring harbor laboratory Press, Cold Spring Harbor, NY.

Schuhmacher, J., Klivenyi, G., Matys, R., Stadler, M., Regiert, T., Hauser, H., Doll, J., Maier- Borst, W. and Zoller, M. (1995) Multistep tumor targeting in nude mice using bispecific antibodies and a gallium chalete suitable for immunoscintigraphy with positron emission tomography. *Cancer Res.*, 55:115.

Schupbach, J., Flepp, M., Pontelli, D., Tomasik, Z., Luthy, R. and Boni, J. (1996) Heat-mediated immune complex dissociation and ELISA signal amplification render antigen p24 detection in plasma as sensitive as HIV-1RNA detection by polymerase chain reaction. *AID*, 10:1085.

Scott, CF., Bolender, S., McIntyre, GD., Holldack, J., Lambert, JM., Venkatesh, YP., Morimoto, C., Ritz, J. and Schlossman, SF. (1989) Activation of human cytolytic cells through CD2/T11: comparison of the requirements for the induction and direction of lysis of tumor targets by T cells and NK cells. *J. Immunol.*, 142:4105.

Scott, JK. and Smith, GP. (1990) Searching for peptide ligands with an epitope library. *Science,* 249:386.

Scott, JK., Loganathen, D., Easley, RB., Gong, X. and Goldstein, IJ. (1992) A faminly of concanavalin A-binding peptide from a hexapeptide epitope library. *Proc. Natl. Acad. Sic.USA*, 89:5398.

Schloer,GM. and JR.Breese S.S. (1982) "Purification of African Malignant Catarrhal Fever Virus Using a Two-phase Aqueous Polymer System. *J. Gen. Virol.* 58:101.

Self, CH. (1985) Enzyme amplification-A general method applied to provide an immunoassisted assay for placental alkaline phosphatase. *J. Immunol. M.*, 76:389.

Shalaby, MR, Shepard, HM, Presta, L, Rodrigues, ML. Beverley, PC., Feldmann, M. and Carter, P. (1992) Development of humanized bispecific antibodies reactive with cytotoxic lymphocytes and tumor cells overexpressing the HER2 protooncogene. *J. Exp.Med.*, 175:217.

Smith, GP. (1985) Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. *Science*, 228:1315.

Smith, TF., Wold, AD., Mark, BA., Espy, MJ. and Marshall, WF. (1993) New developments in the diagnosis of viral disease. *Lab. Diag. Infec. Dis*. 7:183.

Songsivilai, S., Clissold, PM., and Lachmann, PJ. (1989) A novel strategy for producing chimeric bispecific antibodies by gene transfection. *Biochem. biophy. Res.communi.* 164:271.

Sparks, AB., Rider, JE., Hoffman, NG., Fowlkes, DM., Quilliam, LA. and Kay, BK. (1996a) Distinct ligand preferences of SH3 domains from Src, Yes, Abl, cortactin, p53BP2, PLCr, Crk, and Grb2. *Proc. Natl. Acad. Sci. USA*, 93:1540.

Sparks, AB., Adey, NB., Cwirla, S. and Kay, BK. (1996b) In Kay, B.K. Winter, J. and McCafferty's Phage display of peptides and proteins. A library manual. pp227. Academic Press Inc, San Diego, CA.

Staerz, UD. and Bevan, MJ. (1989) Redirecting the celular immune response. *Int Rev Immunol*, 4:159.

Stengele, I., Bross, P., Garces, X., Giray, J. and Rasched, I. (1990) Dissection of functional domains in phage fd adsorption protein: discrimination between attachment and penetration sites. *J.Mol. Biol*., 212:143.

Strappe, PM. Wang, TH. Mckenzie, CA. Lowrie, S. Simmonds, P. and Bell, JE. (1997) Enhancement of immunohistochemical detection of HIV-1 p24 antigen in brain by tyramide signal amplification. *J. Virologi Meth*., 67:103.

Suresh, MR., Cuello, AC. and Milstein, C. (1986a) Bispecific monoclonal antibodies from hybrid hybridomas. *Methods Enzymol*., 121:120.

Suresh, MR., Cuello, AC. and Milstein, C. (1986b) Advantages of bispecific hybridomas in one-step immunochemistry and immunoassays. *Proc. Natl Acad. Sci. USA*, 83:7989.

Suresh, MR.(1991) Immunoassays for cancer-associated carbohyfrate antigens. *Seminars in Cancer Biology*, 2:367.

Takahashi, M. and Fuller, SA. (1988) Production of murine hybrid-hybridomas scereting bispecific monoclonal antibodies for use in urease-based immunoassays. *Clin. Chem*., 34:1693.

Tada, H., Toyoda, Y., and Lwasa, S. (1989) Bisppecific antibody-producing hybrid hybridoma and its use in one-step immunoassays for human lymphotoxin. *Hybridoma* 8: 73

Thomas, F., Smith, AD. Wold, MJ. and Marshall, WF. (1993) New development in the diagnosis of viral disease. *Lab. Dia. Infec. Dis.*, 7:83.

Thirion, S., Motmans, K., Heyligen, H., Janssens, J., Raus, J. and Vandevyver, C. (1996) Mono- and bispecific single chain antibody fragments for cancer therapy. *Eur J Cancer Prev.*, 21:1351.

Uggla, CK., Geisberg, M., Jondal, M. and Knowles RW. (1989) Agnoistic effect of anti-CD2 and anti-CD16 antibodies on human natural killer killing. *Scand J Immunol*; 29: 507.

Valone, FH., Kaufman, PA. Guyre, PM., Lewis, LD., Memoli, V., Deo, Y., Graziano, R., Fisher, JL., Meyer, L. and Mrozek-Orlowski, M. et al. (1995) Phase Ia/Ib trial of bispecific antibody MDX-210 in patients with advanced breast or ovarian cancer that overexpresses the proto-oncogene HER-2/neu. J. Clin.Oncol., 13:2281.

Valadon, P., and Scharff, MD. (1996) Enhancement of ELISAs for screening peptides in epitope phage display libraries. *J. Immuno. M.*, 197:171.

Warnaar, SO., De Paus, V., Lardenoije, R., Machielse, BN., De Graaf, J., Bregonje, M. and Van Haarlem, H. (1994) Purification of bispecific F(ab')2 from murine trioma OC/TR with specificity for CD3 and ovarian cancer. *Hybridoma*, 13:519.

Webb, KS., Ware, JL., Parks, SF., Walther, PJ. and Paulson, DF. (1985) Evidence for a novel hybrid immunotoxin recognizing ricin A-chain by one antigen-combining site and a prostate-restricted antigen by the remaining antigen-combining site: potential for immunotherapy. *Cancer Treatment Rep.*, 69: 663.

Webster, RE. (1996) Biology of the filamentous bacteriophage. In Kay, B.K. Winter, J. and McCafferty's Phage display of peptides and proteins. A library manual. pp9. Academic Press Inc, San Diego, CA.

Wild, D. (1994) Enzymes in immunoassays. In Wild, D. Immunoassay Hand Book. p66. Academic Press Inc, San Diago, CA.

Winter, G. and Milstein C. (1991) Man-made antibodies. *Nature*, 349:293.

Wim, BM., De Lau, AE., Van Loon, KH., Dinko, V. and Bert, J.E.G. B. (1989). Production of hybrid hybridomas based on HAT-neomycin double mutants. *J. Immunol. M.*, 117:1.

Xu, D., Leveugle, B., Kreutz, FT. and Suresh, MR. (1998) Mimetic ligand-based affinity purification of immune complexes and immunoconjugates. *J. Chromatogr. B*, 696:329.